THE CELL SURFACE OF ADHESIVE FRESHWATER BACTERIA

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To my parents - thanks for everything

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Abbreviations

The following additional abbreviations were used in this manuscript:-

Glc. = Glucose; Gal. = Galactose; Suc. = Sucrose; Fru. = Fructose; Tre. = Trehalose; Man. = Mannose; Xyl. = Xylose; Lac. = Lactose; Sta. = Starch; Gly. = Glycerol; Sal. = Salicin; Manl. = Mannitol; Ct. = Citrate; KDO = 2-Keto-3-deoxyoctonic acid. DNA = Deoxyribonucleic acid; RNA = Ribonucleic acid; ATP = Adenosine-tri-phosphate; UDP = Uridine diphosphate. EDTA = Ethylenediaminetetraacetic acid. D = fermenter dilution rate; μmax = maximum specific growth rate.

CONTENTS

ABSTRACT

A number of freshwater isolates were used for this study. Strain S61, a Gram negative adhesive bacterium, was isolated from a local freshwater source. Copious amounts of exopolysaccharide are produced during growth, and play a role in the adhesion process. Analysis of extracellular polymer synthesized by both S61 and other freshwater isolates, revealed that sugars found to occur most frequently were glucose, galactose and mannose; acetate and pyruvate content varied with strain. No unusual components were found in any of the preparations assayed.

A new staining method was developed, allowing the involvement and association of exopolysaccharide in the attachment process to be viewed by light microscopy. Adhesion and biofilm formation was shown to occur in a sigmoidal manner, both *in vitro* and *in situ*. Microcolony development can be prevented by treatments known to affect the secondary and tertiary structure of the exopolysaccharide. However, these treatments have little effect upon cell adhesion.

Following mutagenic treatment, a non-mucoid variant of strain S61 was isolated. Trace quantities of exopolymer were produced, chemically identical to the wild type product. The adhesive properties of the non-mucoid mutant were not impaired; no significant difference in the attached cell numbers occurred compared to the wild type. Microcolony formation however, did not occur.

Continuous culture studies of strain S61 showed that changing both the dilution rate and the culture parameters could markedly influence the cell surface characteristics. Production and viscosity of exopolymers were affected by the growth rate, outer membrane proteins varying with culture temperature. At a low temperature, more variation was observed in the amounts of individual proteins than when grown under different conditions at a higher temperature.

Analysis of purified outer membrane material by affinity chromatography has revealed the presence of a glucose specific receptor, identified as a high molecular weight protein. Less specific receptors for other monosaccharides may also be present. The involvement of specific outer membrane receptors in the initial

stages of attachment was discussed and the possibility of an inter-relationship between polysaccharide synthesis and the availability of receptors for adhesion was considered.

DECLARATION

I hereby declare that this thesis has been composed by myself, and that the work presented herein is, to the best of my knowledge, my own. Any collaborative work has been duly acknowledged in the text.

D G ALLISON

CHAPTER ONE: INTRODUCTION

SECTION 1 General Introduction

The adhesive mechanisms of bacteria have received an increasing amount of attention in recent years. Adhesion can occur on almost any surface in any environment, including those of plants, soil, mouth, gut and aqueous environments. The latter example is important both economically and ecologically, whereas the other examples may all play a role in the pathogenicity of the surface concerned. In any aqueous environment there appears to be a distribution between attached bacteria and the free living (planktonic) population.

Bacteria can vary both in their firmness and in the length of time that they remain attached. The implications of bacterial polysaccharides in the attachment process have been made by various authors. The aim of this review is to investigate bacterial attachment in freshwater, with a view to studying the role of polysaccharides and other cell surface structures in the attachment process.

SECTION 2 The Bacterial Cell Surface

2.1 Surface Layers

Bacteria can be divided into two groups according to the Gram stain. The two groups have very different wall structures; Gram positive bacteria are relatively simple in structure, Gram negative cells are more complex. The cytoplasm of both types is enclosed by the cytoplasmic membrane. Outwith this membrane lies the cell wall which contains features unique to both cell types. Common features are also found, such as peptidoglycan and extracellular polysaccharides. (fig.1).

2.2 The Cytoplasmic Membrane

The cytoplasmic membrane consists almost entirely of protein (45-70%) and lipid (10-35%). A fluid mosaic model proposed by Singer and Nicolson (1972) best describes the lipid bilayer structure discovered earlier (Danielli and Davson, 1935). The lipids are largely polar, predominantly phosphatidylethanolamine in Gram negative bacteria, phosphatidylgycerol and cardiolipin in Gram positive bacteria. Glycolipids have also been observed in Gram positive and in some Gram negative bacteria. (Shaw, 1970).

2.3 Peptidoglycan

All bacteria except the Methanobacteria and Halobacteria contain peptidoglycan as their main structural component. In Gram negative cells, this represents about 5-15% of the cell wall, but may be as much as 90% in Gram positive bacteria (Table 1). Peptidoglycan consists of glycan chains with peptide substituents, the glycan chains containing alternating residues of N-acetylglucosamine and N-acetylmuramic acid in a $\beta(1\rightarrow 4)$ linkage. In Gram negative bacteria the peptidoglycan is located as a separate layer within the periplasmic space (fig.2), whereas in Gram positive bacteria there is some exposed at the cell surface.

2.4 The Gram Negative Cell Wall

In Gram negative bacteria, a second triamellar membrane exisit, to the exterior of the peptidoglycan at the surface of the organism (fig.2). Structurally, the outer membrane consists of phospholipid (26%), protein (11%) and lipopolysaccharide (60%). The 'O' antigen specificity of the bacteria is carried by the LPS.

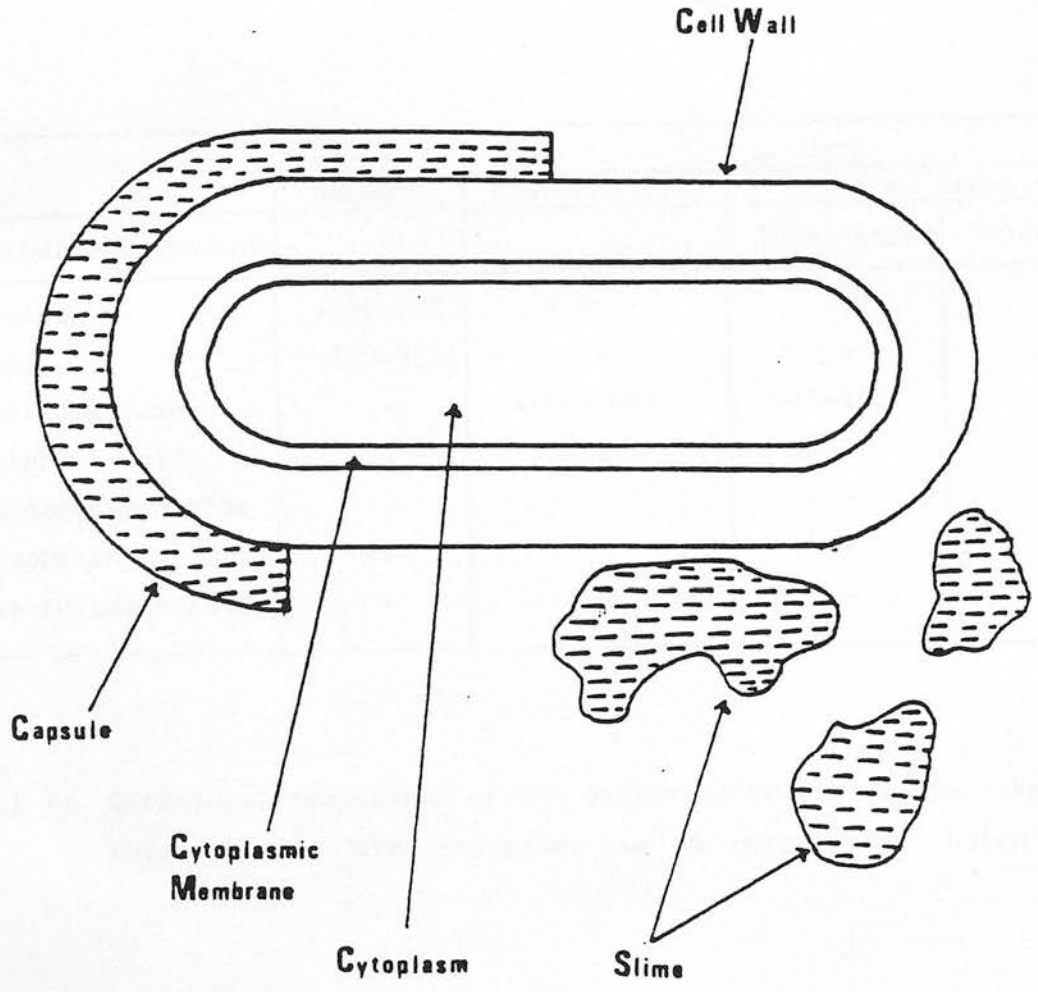


FIGURE 1: The Bacterial Surface

Adapted From Sutherland (1972)

	CYTOPLASMIC MEMBRANE	CELL WALL		
		Gram Positive	Gram Negative	
Structural Components			Inner Layer	Outer Layer
Protein	+(40-70%)	+ or -	-	+
Lipid	+(10-35%)	-	-	+
Peptidoglycan	-	+(40-90%)	+(5-15%)	-
Teichoic Acid	-	+	-	-
Lipopolysaccharide	-	-	-	+
Lipoprotein	-	-	+	+
Lipoteichoic Acid	-	+	-	-

TABLE 1: Structural components of the bacterial cell envelope. Weights, where given, are expressed as % total dry weight.

Included in the outer membrane proteins are the porins which form hydrophilic channels across the membrane, allowing passage of small molecules. Another major protein is the lipoprotein. In the Enterobacteriaceae lipoprotein is present in two forms; 2/3rds are free in the outer membrane and about 1/3rd (the Braun lipoprotein) extends between the outer membrane and the peptidoglycan (Braun, 1978). These are covalently linked to the diaminopimelic acid residues in the peptidoglycan and serve as an anchorage to the outer membrane.

The periplasmic space is bounded by the cytoplasmic membrane and the outer membrane. In addition to the peptidoglycan layer the periplasm contains periplasmic enzymes, binding proteins and pigments. These may be released into the environment when the integrity of the outer membrane is disturbed, permitting enzyme localization studies.

2.5 The Gram Positive Cell Wall

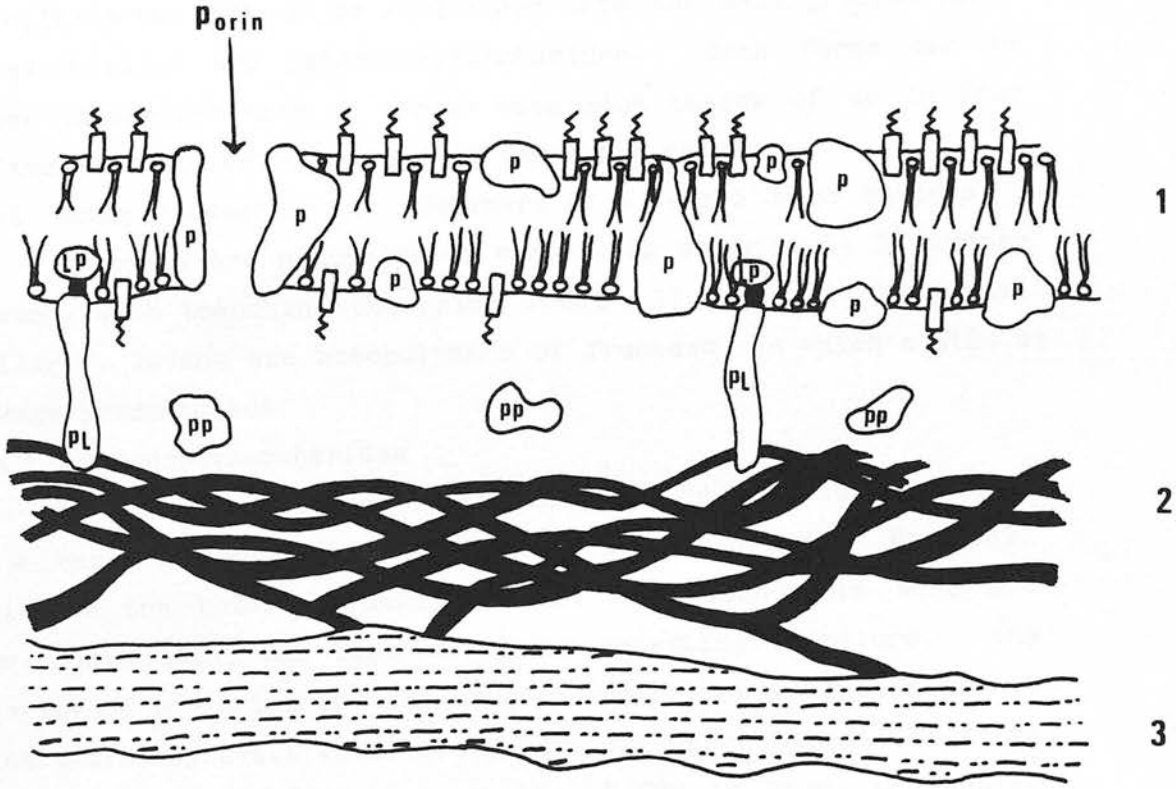
There is no outer membrane in gram positive bacteria. Instead, the thick cell wall is composed of cross linked peptidoglycan covalently linked to highly negatively charged polymers such as teichoic acids and teichuronic acids. Teichoic acids are probably the second most abundant secondary wall polymer isolated from gram positive bacteria. If grown under phosphate deficiency, the teichoic acids are lost and replaced instead by teichuronic acids. Both teichoic acids and teichuronic acids are covalently linked by phosphodiester bonds to between 5 and 10% of the muramyl residues in the peptidoglycan (Ward and Berkeley, 1980).

A second form of teichoic acid, the lipoteichoic acids, are found in the majority of gram positive cells. These are not covalently linked to the peptidoglycan but are intimately associated with the wall, anchoring the polymer to the cytoplasmic membrane.

2.6 Extracellular Polysaccharides

Many bacteria, of both gram denomination, are capable of producing polysaccharides as an additional layer external to the cell (fig.1). They may be excreted as a continuous layer closely associated with the cell surface or as a loose slime excreted freely into the surrounding medium. In each case, their functions towards the cell are similar. Exopolysaccharides are synthesised

FIGURE 2: Schematic Diagram of the Gram Negative Cell Envelope
Adapted From Costerton, Ingram and Cheng (1974)



Key to Symbols:

1	Outer Membrane
2	Periplasmic Space
3	Cytoplasmic Space
P	Protein
PP	Periplasmic Protein
LP	Lipid fraction of Braun's Lipoprotein
PL	Protein Fraction of Braun's Lipoprotein

Lipopolysaccharide

Phospholipid

Peptidoglycan

from a limited range of monomers including hexoses, methyl pentoses, N-acetylated amino sugars and uronic acid. Non sugar redidues have also been reported, eg. formate and succinate, ester linked O-acetyl and ketal linked pyruvate. On the basis of monosaccharide composition the EPS can be subdivided into two main groups, homepolysaccharides and heteropolysaccharides. Both forms can be either linear polymers or linear with side chains of up to four to five monosaccharide units in a repeating sequence.

2.6.1 Homopolysaccharides: Polymers of a Single Sugar Residue.

Dextrans are homopolymers comprising an $\alpha(1\rightarrow 6)$ D glucose backbone with branching occurring at $\alpha(1\rightarrow 3)$ and $\alpha(1\rightarrow 4)$ bonds. Similarly, levans are homopolymers of fructose, in which a $\beta(2\rightarrow 6)$ linkage predominates.

2.6.2 Heteropolysaccharides

Polymers consisting of more than one type of sugar residue in a regular repeating unit, with one exception. Bacterial alginates are heteropolymers composed of D-mannuronic acid and D-guluronic acid, but lack a regular repeating structure. The majority of bacterial EPS are heteropolymers; many contain uronic acids and/or pyruvate ketal groups conferring an overall negative charge to the polymer. Biosynthesis of EPS is known to require the participation of lipid intermediates (Sutherland, 1977) but the nature of the linkage to the underlying wall structures remains unknown.

2.7 Surface Appendages

At the surface of bacteria may be found additional appendages extending into the surrounding medium. These include fimbriae, pili and flagella, reviewed by Sokatch (1979).

SECTION 3 Interfaces Involved in Adhesion

An important consideration to be made when studying adhesion is the role played by the proximity of the substratum. When two immiscible bulk phases come into contact an interface is created. Microbes have been shown to accumulate preferentially at these interfaces (Zobell, 1943; Kjelleberg et al., 1982). Due to their shape and size, bacteria in aqueous systems can be considered as living colloids (Marshall, 1976; Tadros, 1980), and in many cases adhesion can only be described in terms of physicochemical principles (Absalom et al., 1983). This is discussed further in Section 4. Three interfaces of importance exist in the aqueous phase, namely air/aqueous and solid/aqueous.

3.1 Air/Aqueous Interface

Kjelleberg et al., (1982) showed that when nutrient concentrations were too low to support growth in the aqueous phase, growth and division occurred at the air/aqueous interface. The explanation proposed was that nutrients accumulated at the interface through various adsorption mechanisms (including ion exchange), thereby offering a selective advantage over the bacteria in the bulk aqueous phase. Microbial activity in anaerobic sites results in the production of gases such as hydrogen, carbon monoxide, carbon dioxide and methane. These may serve as either energy or carbon sources for other groups of organisms. Ready access to the air/aqueous interface should therefore be of benefit because of the limited solubility and rapid microbial utilization of some of these gases (Marshall, 1976). Other examples of attachment to air/aqueous interfaces, including a 'microflotation technique' are described by Rubin et al., (1966).

3.2 Liquid/Aqueous Interface

Accumulation of bacteria at organic liquid/aqueous interfaces occurs more rapidly and to a greater extent in systems of high interfacial tension than in those of low interfacial tension (Mudd & Mudd, 1924). A perpendicular orientation by certain bacteria at an oil/water interface was noted by Marshall and Cruickshank (1973). They observed that long filaments of Flexibacter CW7 were always directed to the oil/water interface. These findings were

similarly observed at other interfacial systems, prompting the suggestion that the portion of the cell directed towards the interface may be hydrophobic, whereas the majority of the cell in the aqueous phase would be hydrophilic. This non-uniformity of the cell surface may also be found in other systems (eg. the distal end of the holdfast in Caulobacter sp and the surface capsules in Rhizobium trifolii). The existence of compositional differences in the surface polysaccharides of R. trifolii strains is not related to their involvement in a lectin-recognition process (Dazzo, 1980); the saccharide sequence responsible being not necessarily the immunodominant structure of the polysaccharide.

3.3 Solid/Liquid Interface

Solid/liquid interfaces are the most frequently considered interface in the study of microbial adhesion. The characteristics of the substratum, a major component in the attachment process, play an important role. Three main characteristics exist which can influence cellular adhesion.

3.3.1 Physicochemical Factors

3.3.1.1 Surface Charge

Upon immersion, solid surfaces usually acquire a surface charge. This is either through the adsorption of ions from the surrounding fluid or by ionization of surface groups (Fletcher et al., 1980). Homola and James (1977) showed that polystyrene lattices with exposed carboxyl, amino or phosphate groups on their surface were liable to changes in surface charge with changes in the pH of the aqueous phase. In natural waters this surface charge is negative (Neihof & Loeb, 1972; Fletcher & Loeb, 1979). Only when organic material is removed from water, by UV photo-oxidation do surfaces acquire a slightly positive charge (Neihof & Loeb, 1972). Under these circumstances the seven major inorganic ions (Na^+ K^+, Cl^-, CO_3^{2-}, SO_4^{2-}, Mg^{2+} and Ca^{2+}) are responsible for charge on the substratum surface.

As described (Section 2) the surface of the bacterial cell is negatively charged. Thus, in aqueous environments both cell and substratum will be negatively charged. A charged surface has the ability to attract counter ions from the surrounds though this in turn is opposed by the aqueous phase. The end result of

the interactions between electrostatic attraction and thermal repulsion is the diffuse electric double layer surrounding a surface. As illustrated (fig.3) the region next to the charged surface contains a greater concentration of counter ions than in the rest of the aqueous phase. When two diffuse double layers overlap, repulsion of a cell from the substratum surface occurs (Fletcher et al., 1980).

3.3.1.2 Surface Free Energy

The surface free energy is the most important physico-chemical property as it comprises all the surface forces capable of interaction with forces belonging to other phases (Fletcher & Marshall, 1982a). Atoms or molecules on the surface which are able to interact with approaching atoms or molecules provide the free energy of the surface. This can be represented thermodynamically as

$$H = G + T S$$

where H = the total energy of the system, ie. the enthalpy; G = the available or free energy and TS is a function representing the degree of randomness in the system.

To reach an equilibrium value there must be a decrease in free energy so that

$$\Delta G = \Delta H - T\Delta S$$

occurs, ΔG being negative. When ΔG is negative, adhesion is favoured. This occurs when a bacterial surface polymer is adsorbed (Fletcher & Marshall, 1982a). Contact angle measurements have proved to be the most common method of determining the surface free energy, using different liquids on a solid and calculating the critical surface tension (Zisman, 1964). An easier method is to measure the critical surface tension for wetting, ie. wettability (Dexter et al., 1975; Dexter, 1979; Fletcher & Loeb, 1979; Pringle & Fletcher, 1983). This involves measuring the contact angle of water on the substratum and is an indication of their suitability for attachment (fig.4) The more hydrophobic a surface is, the greater the surface tension. The effect of substratum hydrophobicity is discussed later (Section 5.1.3).

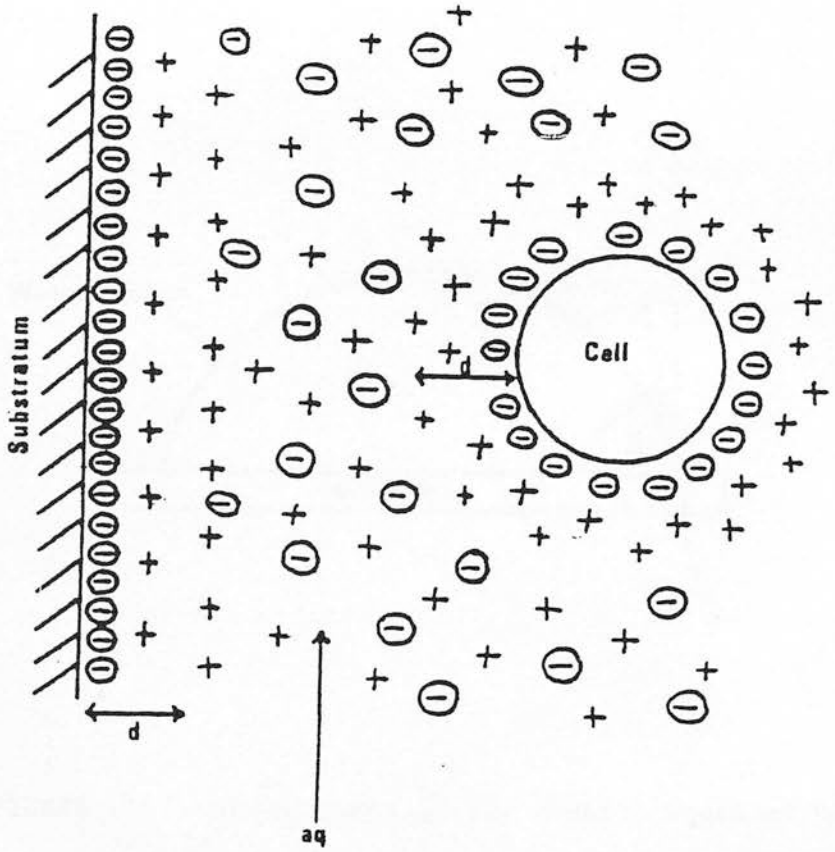


FIGURE 3: The electrical diffuse double layer associated with substratum and cell surface.

Adapted from Fletcher et al.,(1980).

d = Diffuse double layer

aq = Aqueous phase.

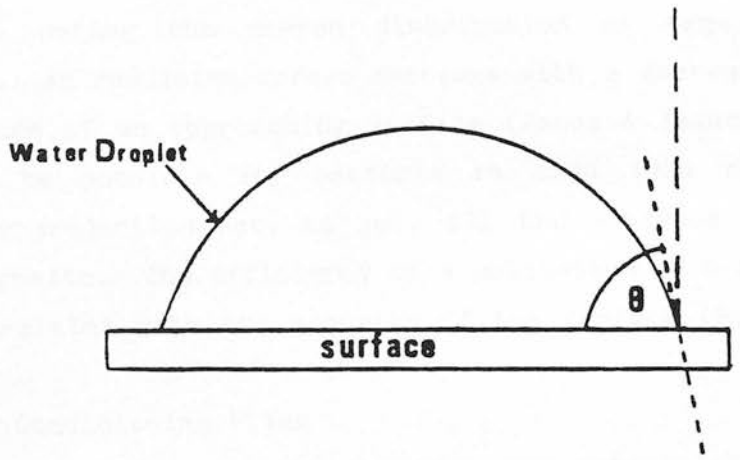


FIGURE 4: Measurement of the contact angle of water.

θ is the contact angle for water. If θ is greater than 15°, the substratum surface is described as hydrophobic. If θ is less than 15°, the substratum is termed hydrophilic.

3.3.2 Physical Factors

Surfaces unless designed specifically, are rarely uniform or flat. With the spreading of a liquid adhesive over a solid, Baier et al.,(1968) observed that contact between the liquid and solid interface was by no means universal, many areas of the surface remaining uncoated. Thus, the adherence of cells can be affected by the topography of the surface. Scanning Electron Micrographs of sand sediments (Weise & Rheinheimer, 1978) revealed less than 5% of the surface populated by bacteria, the greatest cell populations being found in fissures and indentations. Similar results were observed earlier by Meadows and Anderson (1966), noting the uneven distribution of organisms on sand grains. As repulsion forces decrease with a decrease in radii of curvature of an approaching surface (Jones & Isaacson, 1983), it should be possible for bacteria to come into contact with a surface projection but, as yet, all the evidence points towards the opposite. The efficiency of a substratum as a fouling surface is correlated with the porosity of the surface (Pomerat & Weiss, 1946).

3.3.3. Conditioning Films

The negative surface charge and wettability of an inert substratum can influence the process of bacterial attachment, yet those properties are rapidly modified following immersion by adsorption of a conditioning film (Eighmy, et al., 1983; Neihof & Loeb, 1972). In natural waters a varied mixture of macromolecules exist, including proteins, glycoproteins, proteoglycans and polysaccharides (Fletcher & Marshall, 1982a) which will adsorb onto the substratum, possibly to act as a potential nutrient source (Corpe, 1970). The source of these macromolecules may either be autolytic products from the breakdown of dead cells or as secretory products from living cells (Baier, 1972).

Early experiments (Baier, et al., 1968; Baier, 1972) suggested that the conditioning film was in fact a glycoprotein layer. Examination of surfaces suspended in blood revealed a proteinaceous material, 0.5nm thick, present after only five seconds, increasing in thickness to between 10-12nm by 60 seconds after which cells began to adhere. Similar events occurred with

suspended surfaces in saline with adsorption of a glycoprotein layer to a limiting thickness before cellular adhesion. The glycoprotein is similar to that found in marine environments. Thus, a prerequisite for adhesion of any cellular material is the prior accumulation of an organic conditioning film.

Adsorption of glycoproteins occurs irrespective of the original surface properties of the solid phase. The configuration of the glycoprotein may be different on different surfaces, highlighting the original surface properties of the solid (Baier, 1980). The surface charge of the substratum will be modified and be a reflection of the ionogenic groups exposed at the surface of the conditioning film (Marshall, 1980). The overall effect will be to reduce the charge to one of a low negative value (Neihof & Loeb, 1972).

Adsorption of a conditioning film can also alter the surface free energy depending upon the initial state of the surface involved. Dexter et al., (1975) and Dexter (1979) found that low energy surfaces bound macromolecules loosely, whereas high energy surfaces had the adsorbed film more tightly bound. The result of this was that low energy surfaces were more resistant to fouling, and still had some of their original surface exposed. However, Fletcher & Loeb (1979) found the opposite occurring, ie. more cells on low energy surfaces. Using a marine pseudomonad, they found that on a low energy, hydrophobic surface, a covering monolayer formed within 2h, yet on a high energy hydrophilic surface, very low numbers were attached. A wide variety of organisms each with different preferences for high and low energy surfaces has been described (Fletcher, 1980b), strengthening the need for an understanding of the role played by conditioning films on bacterial attachment.

SECTION 4 Attachment of Bacteria

A number of different modes of aquatic bacterial attachment have been described (Corpe, 1970a; Weise & Rheinheimer, 1978). Cell surface structures, described as 'blebs', 'droplets' and fibrils have been observed and thought to play a role in attachment (Corpe *et al.*, 1976). Holdfasts are involved in attachments of prosthecate bacteria such as *Caulobacter* sp (Corpe, 1970a) and fimbriae have also been shown to mediate attachment, though evidence of their significance to attachment in natural aquatic environments is scarce (Fletcher & Marshall, 1982a). More commonly, extracellular polymers forming a continuous coat or "glycocalyx" (Costerton, *et al.*, 1978) around the cell are observed as the adhesive material in aquatic environments.

Attachment can be divided into three main areas on the basis of the cell surface structures involved in the process (fig.5).

4.1 Specific Attachment

Specific attachment involves interactions between complementary molecular configurations on the solid and bacterial surface (Marshall, 1980). These types of interactions are best illustrated by the following examples.

4.1.1 Fimbriae

The adhesive properties of fimbriae have recently been reviewed by Duguid and Old (1980). In the *Enterobacteriaceae* the most common adhesins are the Type 1 fimbriae, attaching cells to the epithelia mucosa (Duguid *et al.*, 1966). Adhesion is inhibited by low concentrations of D-mannose and methyl- α -D-mannoside (Firon *et al.*, 1983; Gaastra & deGraaf, 1982) and thus, because of their protein nature, fimbriae can be considered as lectins. Adhesion inhibition techniques have indicated that all fimbrial receptors recognised to date are carbohydrate (Jones & Isaacson, 1983).

Fimbriated cells display a strong tendency to grow on the surface of static liquid media as a pellicle of aggregated cells (Corpe, 1970). *Pseudomonas*, *Agrobacterium* and *Rhizobium* sp have all been shown to form stars in liquid media at the liquid/air

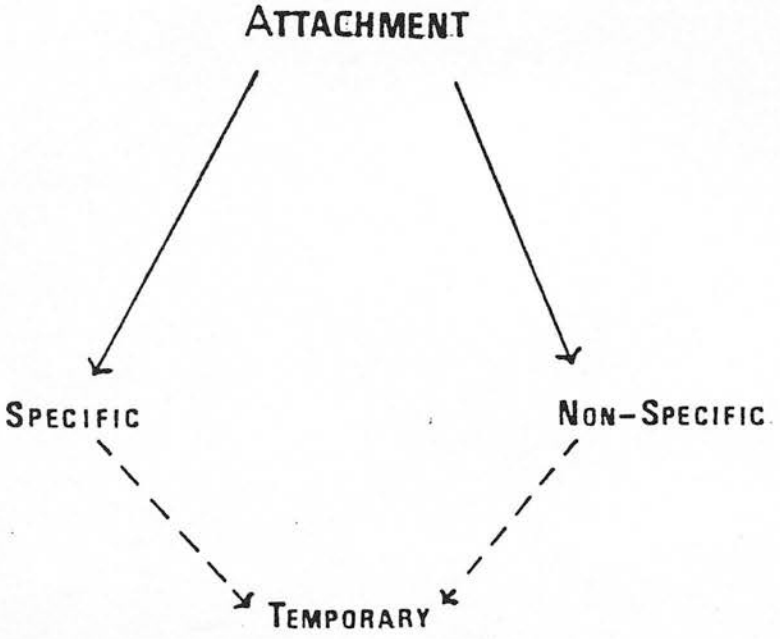


FIGURE 5: Different types of bacterial attachment.

interface (Mayer, 1971). Growth on hexadecane by Acinetobacter calcoaceticus RAG-1 under conditions of low cell density is dependent upon the presence of thin fimbriae mediating adhesion to the hydrophobic surface (Rosenberg et al., 1982). Non-adherent mutants lack fimbriae and are unable to grow on the hydrocarbon. The problems of overcoming electrostatic repulsion are reduced by the presence of fine hair-like probes which have a reduced radii of curvature (see Section 5.2.1).

Pili are frequently described along with fimbriae as a distinction between the two cannot often be made. Generally, fimbriae are about 4μm long with a diameter of 7nm, whereas pili are shorter, between 1-2μm. The adhesion mechanism of pili is similar to that of fimbriae. The gonococcus interacts with the microvillus projections of the host cell surface by action of the pili which penetrate the electrostatic barrier, the host cell receptor comprising of sialic acid and other carbohydrate residues (Watt, 1980). In an extreme environment, Sulfolobus sp have been shown to attach to sulphur deposits in hot acid springs by means of pili (Weiss, 1973). Adhesion is a prerequisite for sulphur oxidation and is an example of reversible attachment (Section 5.1.1).

4.1.2 Holdfasts

Henrici and Johnson (1935) described a number of bacteria which attached to the surface of glass slides, some of which were stalked. These stalked bacteria included species of Hyphomicrobium Blastocaulis and Caulobacter. The wall of the stalk is continuous with the cell wall, and is not a secreted material. At the distal end of the stalk a material is secreted, the holdfast material, which allows adhesion of the cell to occur (Corpe, 1970a). Caulobacter cells can attach in this manner to a variety of solid substrata, themselves, forming rosettes of between 2-100 cells, and may also attach to other microbes, particularly gram positive cells (Poindexter, 1964). A similar mechanism occurs in Hyphomicrobium vulgare (Corpe, 1970a). The chemical nature of the secreted holdfast material is yet unknown, as it is neither a periodate sensitive carbohydrate nor a protein sensitive to trypsin (Poindexter, 1964; Corpe, 1970a). Corpe (1980) observed

that Caulobacter cells became most abundant on glass slides that had been immersed in the sea for a long enough period to be well populated with film forming Pseudomonad sp. The latter may well provide a special surface or specific nutrients, but corroborates Poindexter's (1964) observations that Caulobacter sp attach preferentially to microbial cells than to other surfaces in nature.

4.1.3 Lectins/Antigenic Interactions

Dazzo and Brill (1979) suggested the involvement of a multivalent plant lectin, trifoliin, binding to the acidic heteropolysaccharide isolated from the capsular material of Rhizobium trifolii. The capsular material had 3 polysaccharide components, two of which were antigenic and retained the characteristic specificity for the trifoliin component in the clover root hairs. Electron micrographs (Dazzo, 1980) showed a contact between the fibrillar capsule of the Rhizobium sp and electron dense globular aggregates lying on the outer periphery of the root hair cell wall. These cross reactions were found at discrete sites and are examples of a specific carbohydrate-glycoprotein interaction.

Crown gall tumour infection is caused by Agrobacterium tumefaciens attacking plant cell walls exposed at wound sites. The O-antigen of the bacterial lipopolysaccharide projects externally and interacts with the polygalacturonic acid residues in the plant cell wall (Dazzo, 1980). Avirulent strains have been shown to inhibit virulent strains from infecting a wound, and is due not to a size inhibition, but a surface characteristic which allows them to bind to the infective site in preference to the virulent strains (Lippincott & Lippincott, 1969).

4.1.4 Specific Linkages

The organism Streptococcus mutans produces $\alpha(1\rightarrow6)$ dextrans containing a high percentage of $\alpha(1\rightarrow3)$ linkages from the fermentation of sucrose (Johnson et al., 1975), and is found as a dental plaque in the occlusal fissures of teeth (Gibbons, 1980). Without production of this $\alpha(1\rightarrow3)$ glucan ("mutan") there is a strikingly lower number of attached bacteria (Johnson et al., 1975). Fructan synthesis allows temporary adhesion to the pellicle surface of the tooth, but for permanent plaque formation to occur, there must be mutan production.

4.2 Non-Specific Attachment

In aquatic systems, the most common adhesives are extracellular polymers which form a continuous coat around the cell and are not concentrated in any specific holdfast region. These polymers may include long chain polysaccharides generated by cell surface polymerases as suggested by Costerton et al.,(1978), though no evidence has been published to confirm this. Other possibilities include adhesives produced by goblet shaped wall subunits found in gliding marine bacteria (Ridgway & Lewin, 1973). One of the earliest observations of extracellular polymeric material being involved in adhesion was by Zobell (1943). After staining immersed slides with crystal violet, he described the appearance of microcolonies of attached bacteria surrounded by exopolymers.

The involvement of extracellular polymers in bacterial attachment has been further documented for both freshwater (Jones et al., 1969; Geesey et al., 1977; Sutherland, 1980) and seawater (Corpe, 1970b; Floodgate, 1972; Fletcher & Floodgate 1973; Marshal et al., 1971 a & b). Corpe (1970b) isolated an adhering strain of Pseudomonas atlantica and observed by selective staining using alcian blue that the cells produced, and were embedded in, large quantities of extracellular polysaccharides. Similar findings were observed by Jones et al.,(1969) in an electron microscopy examination of suspended epoxydiscs. Their results revealed that after 7 and 9 days, the suspended discs were covered in an adhering mat of microbes and polysaccharide like material. The mat varied in height between 5 and 8μm, the cells being attached in a similar way to each other and the substratum by polymeric fibrils. Marshall et al.,(1971a) observed that when surfaces with an adherent population of Pseudomonad sp were subjected to shearing cells were removed, leaving a "footprint" of polymeric material on the surface. In all examples studied, the production of polymeric fibrils tends to be more important for the initial colonisation of a surface as cells with specific appendages appear on the surface after periods of more than 24 hours (Section 8).

4.3 Temporary Attachment

As depicted in fig.5, this form of attachment is intermedia-

tory to the other forms previously described. Temporary attachment occurs with the gliding bacteria, Flexibacter spp being a well studied example (Humphrey et al., 1979). Gliding only occurs at a solid/liquid interface, and is a two stage process; i) extrusion of slime followed by ii) the rythmic contraction of waves traversing the entire length of the cell. The slime produced thus gives a high viscosity fluid required for adhesiveness yet allowing translational motion across the surface in an aqueous environment. This describes the situation where the force required for separation of the surfaces is very much greater than the horizontal drag, and is known as Stefan adhesion (Humphrey et al., 1979). Motion is not due to the forceful ejection of slime as the material is secreted ahead of the advancing cells. Most gliding bacteria exhibit the phenomena of attachment by the pole of the cell (Marshall & Cruickshank, 1973), the slime allowing the Flexibacter sp to lift part or all of the cell from the surface. The chemical nature of the slime is that of a water soluble glycoprotein, containing glucose, fucose, galactose and some uronic acids. It is not an acidic polysaccharide, and is similar to the polymer produced by Sphaerotilus spp (Corpe, 1980). The slime is only produced by the Flexibacter sp when on a surface. Vesicular-tubular material has also been observed on the surface of Flexibacter. This is thought to be derived from the lipopolysaccharide membrane, and may play a role in the initial contact with a surface (Ridgway et al., 1975).

SECTION 5 Polysaccharides and Their Involvement in Adhesion

Attachment by extracellular polymeric adhesives has been shown to be a time dependent process (Zobell & Allen, 1935; Zobell, 1943) and is generally thought to occur in three stages, namely reversible adhesion, irreversible adhesion and microcolony formation. The investigations of Marshall et al.,(1971a) supported Zobell's hypothesis of a time dependent process, the main criterion for dividing the process into three stages being whether or not cells were removed from a surface by washing in 2.5% NaCl. Reversibly attached cells were weakly held at the surface still exhibiting Brownian motion, and were easily removed; irreversibly or permanently attached cells do not exhibit Brownian motion and are not easily removed by washing. Irreversible sorption is described as a time-dependent stage due to the synthesis of extracellular polymers which bridge the bacterial and substratum surfaces (Fletcher, 1980a).

5.1 Reversible Attachment

In general terms, reversible attachment is the deposition of cells on a surface. This is an instantaneous attraction of bacteria to the surface where they are weakly held, still exhibiting Brownian motion (Marshall et al., 1971a). Both surfaces as described in Section 3 are negatively charged, which would lead to a repulsion of cell from substratum surface. Attachment therefore depends a lot upon the composition of the aqueous phase. The events occurring at the surfaces within the first few hours are critical and are most likely to be influenced by the surface properties of the substratum (Dexter et al., 1975). Figure 6 represents the forces of attraction and repulsion that exist between two negatively charged surfaces. Cells can overcome the electrostatic repulsion barrier by chemotaxis (Meadows & Anderson, 1968). The action of the flagella may create enough kinetic energy for the cells to overcome the potential energy maximum and subsequently approach the surface at the primary minima. Evidence suggests that flagella are important in cell adhesion (Young & Mitchell, 1972) as removal (Harber et al., 1983; Fletcher, 1980b) leads to a reduction in adhesion. Stanley (1983) showed that adherence decreased at least 90% when flagella

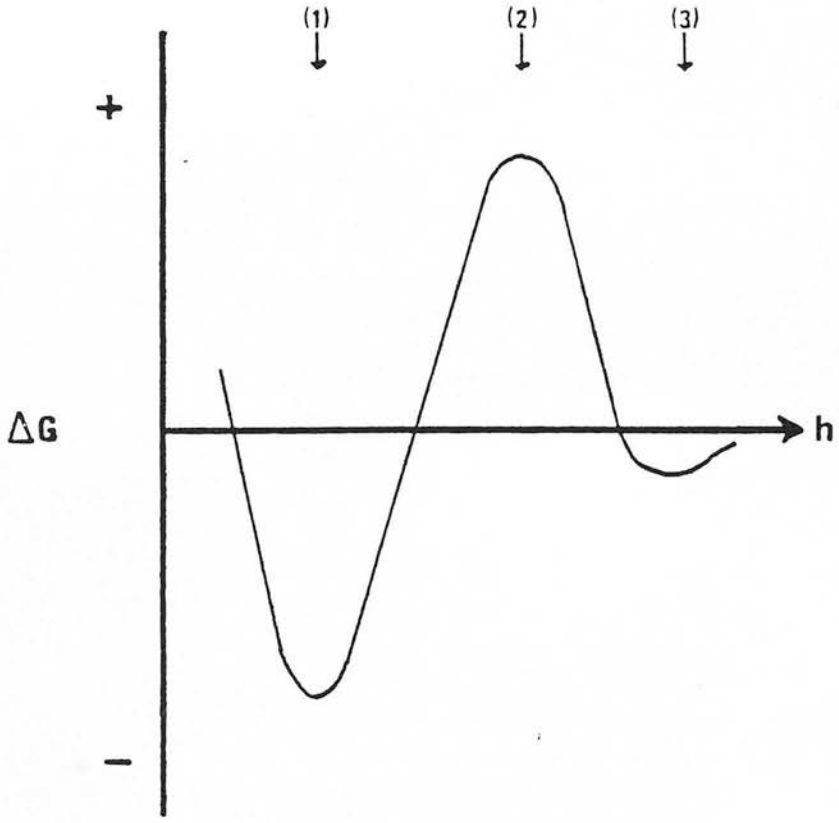


FIGURE 6: Interaction free energy (ΔG) versus particle separation (h) as a representation of bacterial adhesion.

As a bacterium approaches (3), it is attracted by forces of the secondary minimum. Repulsion forces are generated at a closer distance (2), the potential energy maximum. The strong attractive forces created at (1), the primary minimum are associated with irreversible attachment.

Adapted from Jones and Isaacson (1983).

were removed by blending, though this method of removal may lead to loss of other cell surface components. The other advantage of flagella is that they simply increase the statistical chance of a cell 'hitting' the substratum (Fletcher, 1980b).

The most likely method by which cells encounter a surface is by Brownian motion; this only carries cells to within 10nm of the surface before they are repelled. Two principle theories exist to explain attachment, reviewed by Rogers (1980).

5.1.1 D.L.V.O. Theory of Collodial Stability

The D.L.V.O. theory can be considered as a long range force operating at distances up to 10nm, involving London van der Waals forces and the overlapping of electrical double layers. At distances greater than 10nm the forces of attraction are the stronger (as net free energy decreases) and the bodies are held in a state of mutual attraction. The forces of attraction generated at this secondary minimum (fig.6) of potential energy are weak and attachment may be reversed by mild fluid shear (Jones & Isaacson, 1983). The revolving point of this theory is the electrolyte concentration of the surrounding aqueous phase (summarized in fig.7). At a low electrolyte concentration there is a large free energy barrier to overcome, effectively repelling the cell. At a high electrolyte concentration the energy barrier is eliminated and there is a strong net attraction. Finally, at an intermediate electrolyte concentration there is still some energy barrier, therefore, a certain fraction of particle collisions with the surface will result in permanent contacts being made (Rutter & Vincent, 1980; Tadros, 1980). The results of Marshall *et al.*,(1971a) tend to confirm this; with a decreasing electrolyte concentration, they observed a decrease in the reversible sorption of *Achromobacter* sp to glass surfaces, whereas at high electrolyte concentrations a secondary minimum is apparent.

Where the two surfaces bear ionogenic groups of opposite charge, then binding between the surfaces may occur through acid-base type reactions.

The D.L.V.O. theory however cannot fully explain bacterial attachment, as it only considers electrostatic and dipole type interactions that are long range (Maroudas, 1975).

5.1.2 Theory of Short Range Forces

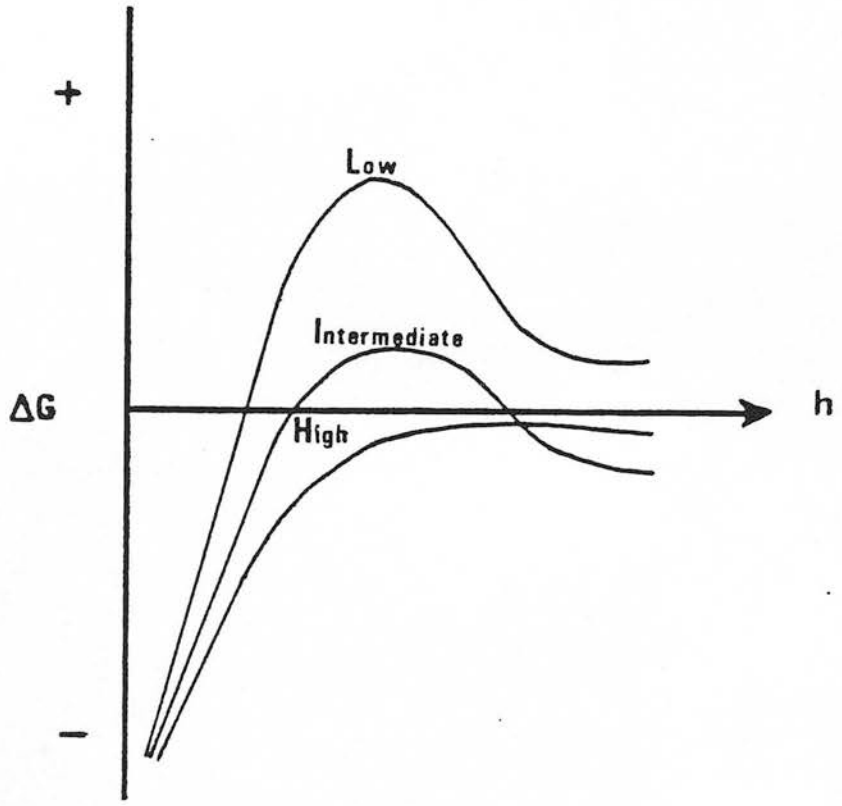


FIGURE 7: Interaction free energy (ΔG) versus particle separation (h) as a function of electrolyte concentration for a charged particle approaching a macroscopic surface of the same sign. As ΔG tends towards a negative value, net attraction occurs.

Adapted from Rutter and Vincent (1980).

As the term suggests, this deals with forces interacting between surfaces less than 0.4nm apart. At shorter distances of separation the adsorbed counter ion clouds on each surface cause the bodies to repel one another (fig.3). If theses forces are overcome and yet smaller distances of separation achieved, then the forces of attraction are again the greater and the bodies are held by the forces at the primary minima. This leads to irreversible attachment, due either to polymer secretion or the presence of fimbriae (Jones & Isaacson, 1983). The short range forces involved include dipole-dipole interactions, ion-dipole, hydrogen bonding, dipole induced interactions and hydrophobic bonds. Table 2 summarizes the type of forces associated with both long and short range interactions. A balance between the short range attractive forces and long range replusive forces is required to hold a cell close to a surface (Fletcher, 1980b).

5.1.3 Hydrophobicity

Recent evidence suggests that a type of interaction occurring in some bacterial attraction processes is hydrophobic bonding. Fletcher & Loeb (1979) and Marshall and Cruickshank (1973) have proposed that there is a clear relationship between the hydrophobicity of an organic substratum and the attachment of a bacterium. Examples confirming this include attachment to siliconized germanium (Marshall, 1976) and to polystyrene (Fletcher & Loeb, 1979; Fletcher, 1980a).

Hydrophobic bonding occurs between two non-ionic and polar surfaces in aqueous solutions. Attraction is weak due in principle to van der Waals forces, but because of the strong interaction between the water-molecules, hydrophobic surfaces are excluded from the aqueous phase. Hydrophobicity is correlated with the water contact angle on a substratum (fig.4); the greater the critical surface tension, the greater the degree of hydrophobicity. When the surface tension of the suspending liquid is greater than that of the bacteria, adhesion is more extensive to hydrophobic surfaces (Absolom et al., 1983). However, the opposite pattern prevails when the surface tension of the bacteria is greater than that of the suspending fluid.

The results expressed by Dexter et al.,(1975) conflict with

TABLE 2

Forces of Attraction and Repulsion Between Microbial Cells and Inert Surfaces

Forces of Attraction

1 Long Range

Charge fluctuations.

Electrostatic attraction between surfaces of similar charge.

London van der Waals forces of attraction.

Gravitational forces.

Positive Chemotaxis (cellular mobility).

Polar interactions.

2 Short Range

Chemical bonds (hydrogen, thio, amide, ester, electrostatic, covalent).

Dipole interactions (dipole-dipole, dipole-induced-dipole, iondipole).

Hydrophobic bonding.

Interparticle bonding.

Hydronamic forces.

Ion pair formation ($-NH_3^+ \ldots\ ^-OOC-$)

Ion triplet formation ($-COO^- \ldots Ca^{2a} \ldots ^-OOC-$)

Forces of Repulsion

Charge repulsion between surfaces of similar charge.

van der Waals forces of repulsion.

Steric exclusion (hindrance).

Negative chemotaxis (cellular mobility).

Adapted from Daniels (1980); Tadros (1980).

these findings. They reported higher numbers attached to glass and other hydrophilic surfaces and only a rise in attachment on very hydrophobic surfaces such as polytetrafluoroethylene. These discrepencies could be due to the alteration of the substrata through the adsorption of polymers, particularly proteins. Hydrophobic attraction itself may provide only a means of reversible sorption to solid surfaces, additional mechanisms being required for further adhesion.

5.2 Irreversible Attachment

Irreversible attachment occurs when the bacterium-surface binding resists strong washing, the forces of attraction at the primary minima being greater than those of repulsion. This is achieved by a decrease in the radii of curvature associated with polymeric fibrils and fimbriae (Fletcher & Marshall, 1982a). Both energies of attraction and repulsion are functions of the radii of curvature of the bodies. The magnitudes of both decrease with decreasing radii of the bodies, those of repulsion decreasing more (Jones & Isaacson, 1983).

The involvement of polymeric fibrils bridging cell to surface has been observed on a variety of surfaces; on detritus (Paerl, 1975), Cu_2O paint (Dempsey, 1981a & b), sand (Weise & Rheiheimer, 1978), the common feature being the gradual build-up of small amounts of polymeric material into larger matrices. A complicating factor is the question as to whether or not the polymer is formed before attachment or produced during reversible attachment (Fletcher & Marshall, 1982a). Evidence suggesting that polymer synthesis was needed for firm attachment was presented by Gibbons and van Houte (1975), obtained from studies with Streptococcus mutans. Polymer synthesis may not be necessary for initial adsorption, but the production of extracellular glucans from sucrose can strengthen the attachment of weakly held cells and lead to plaque formation. Further evidence (Powell & Slater, 1982; Eighmy et al., 1983) also suggests that polymer build-up occurs after bacterial adsorption. An elegant study by Brown et al., (1977) indicated that greater attachment occurs when only polymeric fibrils are produced by cells than when a gelatinous matrix is produced. Saturation of specific binding sites may be

the reason for this (Wardell et al., 1980; Pringle et al., 1983).

As the bacterial film develops, more substantial amounts of polymer are involved until the organisms growing on a surface are surrounded by a reticulum of polymeric fibrils facilitating cell-cell and cell-surface bridging (Corpe, 1964; Jone et al., 1969; Fletcher & Floodgate, 1973; Geesey et al., 1977). Fletcher and Floodgate (1973) observed the involvement of a primary and secondary polysaccharide in attachment. Using Transmission Electron microscopy, they observed that the primary polysaccharide was an electron dense layer found on both suspended and attached bacteria and is involved in the reversible attachment of cell to surface. The secondary polysaccharide however only appears on attached cells and is thought to be derived from the primary polysaccharide. It is a fibrous, reticular substance that is involved in irreversible attachment (Floodgate, 1972) and is similar to the matrix observed by Jones et al.,(1969).

Proteins may also be involved in the irreversible attachment of cells, as the action of certain proteases (pronase, trypsin) have been shown to disrupt attachment (Fletcher & Marshall, 1982b; Danielsson et al., 1977). Because of the presence of both polysaccharides and proteins on the bacterial surface, the cell is equipped for a range of polar and electrostatic interactions as well as hydrogen bonding.

In some situations, reversible sorption is not a prerequisite for irreversible sorption and firm attachment can occur quite spontaneously, for example, adhesion of certain marine bacteria to polystyrene and similar plastics (Fletcher, 1980a).

5.3 Microcolony Formation

After single cells have attached firmly, growth and division rapidly leads to the development of microcolonies (Wardell et al., 1980). As they enlarge, they coalesce to form a layer of cells covering the surface. Large amounts of extracellular polysaccharides may also be produced which embed the cells in a polymeric matrix (Jones et al., 1969; Geesey et al., 1977). Once a thick slime has built up there is often a sloughing off of the surface cells which can subsequently act as an inoculum for the aqueous phase (fig.8). Biofilm production is the net accumulation of attached

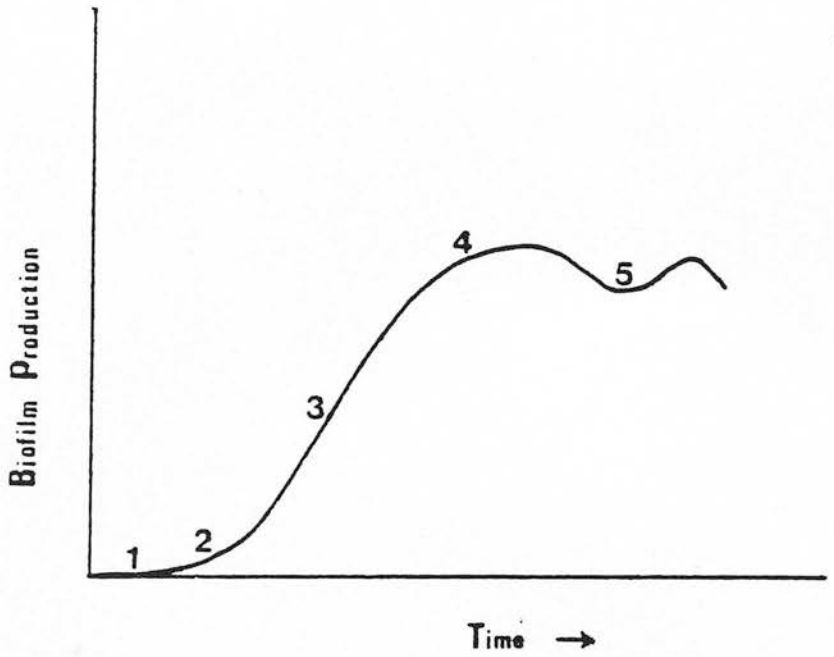


FIGURE 8: Biofilm formation on a solid surface as a function of time.

1 Reversible attachment.

2 Irreversible attachment.

3 Microcolony formation and biofilm production.

4 Detachment.

5 "Sloughing" off.

Adapted from Bryers and Characklis (1982).

material due to cellular reproduction and microbial production of EPS (Bryers & Characklis, 1982). Detachment is the continuous loss of relatively small amounts of biofilm material from the surface and "sloughing" is the random, massive removal of biofilm attributed to oxygen and/or nutrient limitations within the biofilm (Bryers & Characklis, 1982). The extent of polysaccharide formation observed would indicate considerable energy outlay by the attached microbial cells. In laboratory studies the extent of converstion of substrate to EPS varies greatly with the species and the growth conditions (Sutherland, 1983a). Xanthomonas campestris strains grown under varying nutrient limitations with glucose as the carbon source had conversion yields between 27.5% and 82% (Sutherland, 1983b).

As microcolonies enlarge, they do so along the plane of the surface until they reach a certain size whereupon development takes place away from the surface. Eighmy et al., (1983) observed the involvement of at least three different types of polymeric matrices in the formation of wastewater biofilm. Over a 144h sampling period a variety of actively dividing morphological types were shown to be associated with the polymeric adhesives. In many laboratory studies microcolony formation is the build-up of a mono-layer of cells whereas in the natural environment build-up would be due to a mixture of organisms.

Microcolony formation does not occur all of the time. After adsorption to exposed surfaces many micro-organisms fail to develop into microcolonies (Brock, 1971) and many species of bacteria such as Caulobacter sp are lost when cell division occurs (Sutherland, 1983a).

5.4 Properties of Adhesive Polysaccharides

Few of the polysaccharides associated with bacterial adhesion have been analysed in an attempt to relate structure to adhesiveness. Difficulties arise when trying to extrapolate laboratory results into meaningful interpretations of the natural environment, hence the majority of adherent polysaccharides are recognised through histological and EM techniques. Sutherland (1980 & 1983a) investigated the chemical and physical properties of polysaccharides isolated from adherent aqueous systems along

with the effect of ions upon these polymers. The results showed that D-mannose, D-glucose and D-galactose occurred most frequently with all the polymers appearing to contain a uronic acid. Physical properties indicated that solutions of most of the polymers showed pseudoplastic characteristics. There was an absence of any appreciable ion interaction with freshwater polymers, however, the polymers from several of the seawater isolates formed gels or precipitates with both divalent and trivalent cations.

Similar chemical studies by Corpe (1970b) again showed the presence of D-mannose, D-glucose and D-galactose along with D-galactosamine and pyruvate in the polymer isolated from a Pseudomonas atlantica strain. A high uronic acid content was found (hexose: uronic acid = 1:1) but no protein. Fletcher (1980a) however, isolated a polymer from an adhered marine Pseudomonas sp which contained protein ranging from 50-80% of the dry weight. This illustrates the fact that no single chemotype is responsible for adhesion, there being an apparent absence of any unusual components or rare monomers in the polymers concerned.

SECTION 6 Parameters Affecting Attachment

The quantity and quality of polymers produced by different bacterial strains varies considerably. Because of this, different bacteria may also vary in their ability and strength of attachment. Variations not only occur between species, but also within species (Hartley et al., 1978). The attachment tendencies of any bacterial strain are influenced by the environmental conditions as well as the physiological state of the organism. These parameter differencies are discussed below.

6.1 Temperature

As the temperature increases, the number of permanently attached cells also increases (Fletcher, 1977 Minato & Suto, 1979). Pedersen (1982) observed that when glass slides were inserted into a biofilm reactor at either 2-7°C or 15°C, the rate of biofilm accumulation was 5.6 times slower at the lower temperature. Explanations proposed have included that 1) at low temperatures, the attachment efficiency is lowered because of an increase in viscosity of the media or of the bacterial surface polymer (Hattori & Hattori, 1963); 2) higher temperatures favour chemisorption which is a physicochemical response; 3) the temperature differences may affect the physiology of the organisms (Fletcher, 1977). Although these explanations appear well defined, the actual adhesive process is not, though the results are similar to those predicted by models of molecular adsorption from solutions to surfaces.

6.2 Dissolved Material

6.2.1 Inorganic Material

Dissolved substances in the surrounding media can significantly affect attachment through their influence on interactions between the bacterial and substratum surface. The electrolyte concentration has been shown by various groups (Marshall et al., 1971a ; Fletcher, 1979; Marshall, 1972) to play an important part in cell attachment. Marshall et al., (1971a) observed that a marine pseudomonad was prevented from attaching to a surface by the omission of Ca^{2+} and Mg^{2+} ions from the media. Similarly, biofilm attached to a millipore filter was severely disrupted when

transferred to Ca^{2+} and Mg^{2+} deficient media (Fletcher & Floodgate, 1976). It was suggested that the divalent cations are important in the maintenance of the structural integrity of the intercellular polymeric matrix within the attached microcolonies by possibly cross linking or screening acidic groups on the adhesive (Fletcher, 1980a). Fletcher and Floodgate (1976) and Fletcher (1979) suggested the involvement of a secondary polysaccharide, found only in attached cells, being disrupted by the cation deficiency, whereas the primary polysaccharide found on all cell types remains intact. The evidence to support this idea though is based solely on TEM studies and should be treated with caution.

Bacteria have been shown to be reversibly sorbed at lower concentrations of a divalent electrolyte than of a monovalent electrolyte, an effect clearly related to the greater compression of the double layer in the divalent system at comparable concentrations (Marshall, 1972). A similar progression would be expected by the addition of trivalent ions to the system. In practice however, the opposite occurs (Fletcher, 1979). Addition of Al^{3+} and La^{3+} inhibited the attachment of a marine pseudomonad presumably by combining with the negatively charged sites on the adhesive and displacing the divalent cations (Fletcher, 1980a).

6.2.2 Organic Material

Fletcher (1976) tested the effect of a variety of proteins on the ability of a marine pseudomonad to attach to a polystyrene surface and suggested that a relationship existed between the attachment process and the concentration of dissolved organic material present in the medium. The most probable explanation is that the inhibitory effect is due to steric exclusion.

The observation (Fletcher, 1976) that basic proteins did not inhibit attachment contradict the report of Meadows (1971) who found that the basic proteins tested did not reduce attachment. The differences in results can be due to the different cell types and surfaces used, indicating again the complex nature of the attachment process.

6.2.3 Micro-organic Material

Growth of pseudomonad cultures at a low glucose concentration, $7mgL^{-1}$, stimulated sorption to surfaces, whereas at $14\text{-}21mgL^{-1}$,

sorption was reduced and completely inhibited at 30 and 70mgL^{-1} (Marshall et al., 1971a). Similar results were confirmed by Brown et al.,(1977) using a carbon limited chemostat. In an attempt to relate laboratory findings to in situ conditions, they observed that although small amounts of polymeric film was being produced, cells were adhering in greater numbers than when conditions led to much EPS being produced. Consequently, specific cell surface receptors were suggested as being involved in the initial adhesive step.

6.3 Culture Concentration

In concentrated cell suspensions continuous monolayers can be formed rapidly, ie. within 10-30 min. (Characklis, 1973). There is a positive correlation between the number of attached cells and the increase in cell culture concentration. When the surface becomes saturated, the numbers level off. With a low concentration of cells a steeper increase in the number of attached cells occurs than at a higher cell concentration (Fletcher, 1977). This is due to the increased chance of hitting the surface thereby making contact. The longer that cells are left to approach a surface, the greater the number that adhere.

6.4 Culture Age

It is generally found that as cells get older attachment decreases (Zobell, 1943; Marshall et al., 1971a; Fletcher, 1979). Fletcher (1977) observed that with log phase cells a polystyrene surface was saturated within 2.5-3 hours, whereas with stationary phase cells this was extended to between 3.5 and 4 hours. Two factors have been proposed that probably influence this:-

a) Changes in cell motility

b) Changes in the quality or quantity of cell surface polymers produced (Fletcher, 1977). In most cases, micro-organisms tend to aggregate under conditions of declining growth as this is when natural polymers are usually excreted or exposed at the surface (Harris & Mitchell, 1973).

6.5 pH

Wilkinson and Hamer (1974) showed that with a mixed culture growing on methane, there was considerable attachment to glass and stainless steel surfaces at pH values in excess of 5.8. No such

attachment occurred when the pH was at 5.8 or below. Similarly, cells that attached at pH 6.2 were found to detach and flocculate upon subsequent adjustment to pH 5.7. This trend has been observed by other workers (Fletcher & Floodgate, 1973; Stanley, 1983) and complements the earlier studies of Wilkinson (1958) who reported that EPS production is maximum when the pH is near neutrality. Fletcher and Floodgate (1973) suggest the prevention of primary EPS appearing on preparations of attached bacteria at pH 8.6 but again fail to produce any quantitative evidence to confirm this. The effect of pH upon the cell surface can be studied using an ion exchange column (Wood, 1980a), the results indicating a specific adsorption at pH values between 5 and 8.

6.6 Shear Rate

Estimations of shear rates applied in the natural environment can only be approximations, the shear rates being likely to vary considerably. Primary film formation is retarded by high velocities, but after it is formed, faster flow rates lead to greater growth. This greater growth is probably due to reduced boundary layer thickness and thus better transport of substrate from the bulk fluid to the micro-organisms. Because of this, Characklis and Cooksey (1984) suggest that the flow regime be carefully defined (ie either quiescent, laminar or turbular). At a low flow rate, about 40% saturation of the surface occurs, whereas at a laminar and fast flow rate, about 1% and 0.1% saturation occurs, respectively. In slow moving fluids the shear rate is too slow to stop the formation of adhesive bonds, allowing a high number of cells to adhere (Sutherland, 1983a). At faster flow rates the shear generated is such that most cells are unable to resist its action. Slimes produced at high velocities however adhere more firmly to the surface than those produced at lower rates.

SECTION 7 Methods of Study

7.1 Light Microscopy

Demonstration of an adsorbed microbial population by cell removal and counting is best accompanied by some form of microscopic examination. The main value of light microscopy would appear to lie with enumeration studies (Henrici, 1932; Zobell, 1943; Eighmy et al., 1983) indicating the different morphological types present (Corpe et al., 1976) and their distribution on the surface (Meadows & Anderson, 1966). Phase microscopy can be used to examine living bacterial populations on surfaces and has the advantage that cells do not need to be fixed or stained. The adsorption process can be studied by phase, but little information is provided about the role of any polysaccharide present. Zobell (1943) used methylene blue, gentian violet and carbol fuchsin stains on immersed slides, and observed microcolony formation covering 1/50th to 1/10th of the surface, associated with an irregular film of stained material. The use of stains thought to demonstrate carbohydrate material, such as alcian blue and periodic acid schiff, resulted in the idea that carbohydrate was always present in microbial aggregates. These staining methods do not provide any significant knowledge about the actual carbohydrate-containing material present (Sutherland, 1983a). The main disadvantages of light microscopy are the distinctly limited number of optically suitable substrates and the low resolution which is insufficient to reveal attachment mechanisms.

7.2 Electron Microscopy

Both Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) can provide clear evidence that polymer bridging is a principal mechanism in bacterial attachment.

7.2.1 Transmission Electron Microscopy

The advent of ruthenium red staining has allowed the visualization of highly hydrated non electron dense materials by the TEM. The role carried out by these EPS can now be recognized, whether it be for temporary adhesion in gliding bacteria (Humphrey et al., 1979) or for enclosing dense populations of microbes adhering to inert surfaces (Geesey et al., 1977). The fibres enmeshing the attached populations are thought to be derived from the bacteria, being secreted by the cells within. TEM is best

carried out with immersed grids and examined by negative staining, but is not so good with sections of embedded material as it yields little information relating to the distribution of bacteria on the surface. Colonisation of tissues is well described (McCowan et al., 1978). TEM showed the presence of both the glycocalyx of bovine epithelial cells and fibrous carbohydrate coats surrounding the adherent bacteria, the carbohydrate coat mediating attachment between themselves and epithelium, to food particles and to each other as microcolonies were formed.

Secondary organization is well detected by TEM as it provides a sectional image at right angles to the colonized surface. The polysaccharide stretching between adhering pseudomonad cells and a millipore filter was noted as a layer 15-25nm thick (Fletcher & Floodgate, 1973). A second polymer, also staining with ruthenium red, was formed after the cells were attached and differed in appearance from the initial polymer. Three distinct 'glycocalyces' were positively stained by ruthenium red in a study of wastewater biofilm (Eighmy et al., 1983). In all cases the polymeric coat appeared to mediate cell attachment.

Ruthenium red does not necessarily indicate the presence of an acidic polysaccharide (Sutherland, 1983a). Neutral polymers such as the α1,3 glucan produced by S.mutans (Abbot et al., 1980) can take up the stain, whereas some acidic polymers (eg. hyaluronic acid) fail to stain with ruthenium red (Sutherland, 1983a). Although ultrastructural characterization of EPS has been informative, interpretations must be viewed with great caution. Virtually all TEM observations are based on the dehydration of specimens which can be expected to produce substantial alterations in EPS structure (Geesey, 1982). Inclusion of antibody stabilization steps during specimen preparation offers some hope in solving this uncertainty (Mackie et al., 1979).

7.2.2 Scanning Electron Microscopy

The majority of recent studies have used SEM as a means of examining the adherent population. This technique offers a 'face' view of the surfaces and easily relates the distribution of bacteria to the topography of the surface in question. Bacteria adhering by polymeric fibrils were found in depressions between epidermal

cells on the surfaces of leaves (Leben & Whitmayer, 1979) (Plate 1) and an examination of sand grains revealed that less than 5% of the total surface was populated by cells (Weise & Rheinheimer, 1978). Polymer was observed associated with cells of different morphological types and could be seen as strands of varying thickness. Improvements in preparatory techniques have minimized the drying-related collapse of tissues and of adsorbed bacteria by the use of critical point drying (Costerton, 1980). Elimination of the heavy metal impregnation techniques has meant that fine structures previously buried during metal deposition can now be observed. Examination by such methods have revealed adherent bacteria associated with slime filaments or secretions (Rheinheimer, 1980). Wooden surfaces(Cundell & Mitchell, 1977), painted slides (Dempsey, 1981b) or detritus particles (Paerl, 1975) have all revealed an extensive weblike network of slime material intimately associated with the attached bacterial community. These results tend to confirm the high incidence of polymer strands or capsular material associated with attached microbes, and are probably involved in the anchoring of cell to substratum. SEM with its large depth of focus provides a three dimensional picture of the adherent population, and is limited only in its inability to detect bacteria colonizing interior surfaces.

Although EM studies have suggested that adhesion involves polymeric material completely surrounding the cell, this is not always true. Studies by Marshall & Cruickshank (1973) have revealed that rosette forming species adhere by means of polymer sited at the pole of each cell. The result is that they are orientated at right angles to the solid surface. How frequently this occurs in the natural environment is open to investigation.

7.3 Experimental Evidence

Although microscopical information indicates the involvement of polymeric fibrils in the attachment process, the nature of the adhesive involved in the primary attachment is still unknown. Treatments affecting the integrity of the cell surface have been shown to disrupt adhesion (Meadows, 1971). When cells were heated to 65^{o}C for 10 min. or treated with 2% formalin, attachment was reduced. Cells killed by UV irradiation may still attach as the cell surface has not been altered by the treatment.

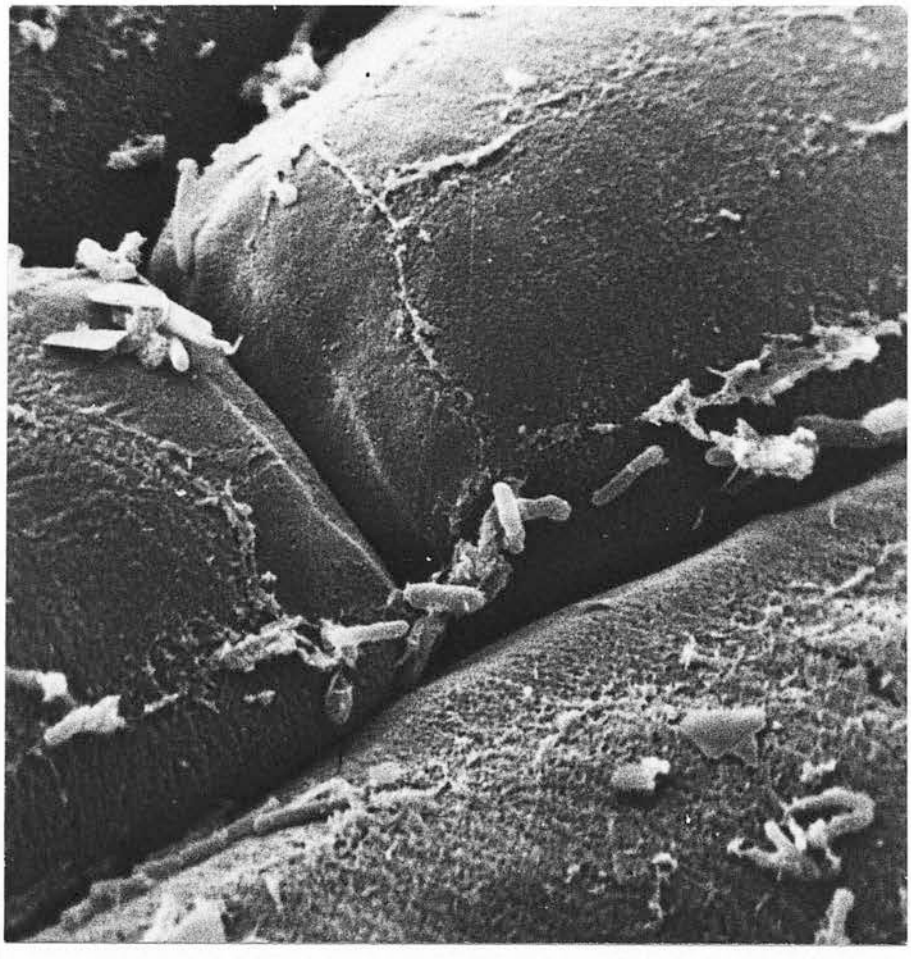


Plate 1: Bacteria adhering to Birch leaf epidermal cells.
From Aitken, (1984)

Conflicting reports suggest that the initial adhesive is either a polysaccharide or a protein. Fletcher and Marshall (1982b) suggested that a protein was involved as the action of pronase inhibited adhesion of the Pseudomonad species. Inclusion of the antibiotic chloramphenicol into the culture media (Marshall, 1972; Fletcher, 1980b) prevented the cells tested from adhering. As chloramphenicol inhibits the synthesis of proteins, the evidence suggests that proteinaceous material is involved in attachment. Further evidence suggesting a protein adhesin was submitted by Danielsson et al.,(1977). Pronase and trypsin both reduced attached cell numbers (50% loss in 5 min., 50% in 30 min., respectively) indicating proteinaceous material. Polysaccharases were similarly tested, lysozyme having a weak effect (10% loss in 30 min.) and amylase having no effect at all.

In a comparison of mucoid and non-mucoid mutants (Pringle et al., 1983), the non-mucoid variants showed lower levels of attachment compared to the wild type. As no significant changes could be observed in the outer membrane profile, the inhibitory effect is probably due to the saturation of cell binding sites previously discussed (Brown et al., 1977).

Evidence for a polysaccharide-like material being involved in adhesion has arisen from chemical studies. Addition of sodium periodate to the culture medium resulted in a rapid desorption of cells from a glass surface (fig.9), whereas attempts at removal by enzymic treatments proved unsuccessful (Marshall & Cruickshank, 1973). A similar treatment with periodate resulted in the disruption of the cell coat of adhering Ruminococcus sp., leaving only fragments on the cell surface (Latham et al., 1978). Fletcher (1980a) suggested that a carbohydrate adhesive may be involved by treatment of both free living cells and attached cells with sodium periodate and disodium tetraborate. Both were found to inhibit or disrupt cell adhesion. The use of metabolic inhibitors to prevent energy production can also disrupt adhesion, indicating that the process is energy dependent (Fletcher, 1980b).

What seems clear is that the primary adhesin may vary between different organisms or even between similar species, depending upon the nature of the substratum. A recent paper by

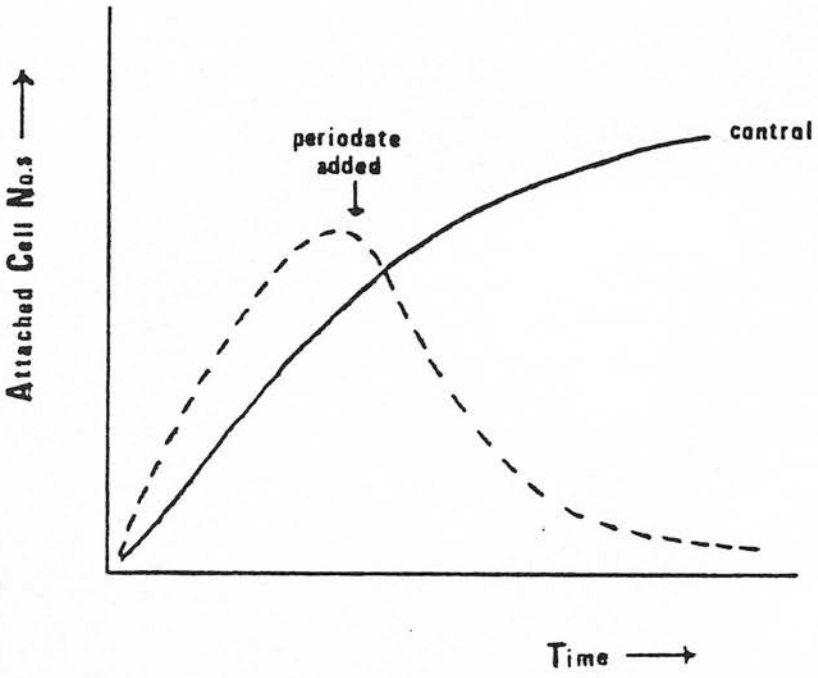


FIGURE 9: The effect of sodium periodate upon sorbed cell numbers.

Adapted from Marshall and Cruickshank (1973).

Moore and Marshall (1981) has indicated that a glycoprotein or peptidopolysaccharide may be the adhesive material that causes rosette formation in Hyphomicrobium sp, and may well be involved in other cellular interactions.

7.4 Continuous Culture

The investigation of bacterial adhesion within a chemostat is not new. A number of studies have used continuous culture to examine adherent organisms and to create conditions that resemble as closely as possible those existing in the natural environment. Brown et al.,(1977) reported on the effect of varying the nature of the limiting substrate on the colonization of aluminium foil by bacteria enriched from river water. This has been further discussed by Wardell et al.,(1980) and in addition, the effects of wall growth and colonization of surfaces within the chemostat have been described (Larsen & Dimmick, 1964; Topiwala & Hamer, 1971; Wilkinson & Hamer, 1974). A mathematical model has been proposed to show the effect of wall growth in a single stage chemostat (Topiwala & Hamer, 1971). Three assumptions are made. 1) Bacteria adhering to the walls of the vessel in contact with the culture form a monolayer. 2) After an initial build up, the total mass adhering remains constant. 3) The growth rate of attached bacteria is the same as that of the suspended population. Experimental results on the effects of wall growth by methane-utilizing bacteria closely parallel the above model (Wilkinson & Hamer, 1974). Differences arise though, due to bacteria not growing as monolayers (Munsen & Bridges, 1964) and the thickness of the film varying (larsen & Dimmick, 1964).
Molin (1983) pointed out that the maximum specific growth rate, μmax, cannot be measured by a washout method if the organism has a tendency towards biofilm formation. Instead, it was suggested that the maximal growth capacity be related to μmax and estimated from the curve of biomass against dilution rate. The point where biomass starts to decrease from its maximum value being taken as the maximum growth capacity of the culture.

The metabolic activity of adsorbed cells has been shown by Hendricks (1974) to be greater than the cells in suspension. The population adsorbed to the glass surface multiplied and appeared to form two distinct layers. One layer was easily removed while

the other adhered strongly. These results resemble findings from other workers using marine aqueous systems (Marshall et al., 1971a). Zobell (1943) indicated that as the total available surface area increased, bacterial activity increased. In the continuous culture of bacteria, the use of glass beads and glass wool to increase the surface area resulted in a 30 fold increase in cell productivity (Larsen & Dimmick, 1964). Of the strains tested, some showed as much as 90% of the population attached, while in others this did not occur. Thus, wall growth can significantly influence the population density and kinetics of continuous growth.

The chemostat has the advantage of being able to follow the growth and attachment of cultures over an extended time period. This permits physiological studies to be carried out on the organisms under varying limitations (Molin & Nilsson, 1983) and also allows different surfaces to be suspended for varying periods of time. Such studies have indicated that under both carbon and nitrogen limited conditions the surface growth was accompanied by the production of polymeric microfibrils which were associated both with cell-surface and cell-cell adhesion (Brown et al., 1977); Ellwood et al., 1982). Under the conditions tested by Molin and Nilsson (1983), only alteration of the pH value (between 5.5 and 6.7) had any significant effect on biofilm production without affecting the suspended cells.

The potential offered by steady state continuous cultures to investigate cell growth and attachment, biofilm production and mixed cell interactions has as yet not been exploited to its full extent.

SECTION 8 Microbial Species Involved in Adhesion

8.1 Bacterial Species

Very few bacteria observed to be attached by polymeric adhesives have been characterized beyond the gram stain. A large variety of types exist and their lack of specific economic importance prevents any further identification. Morphological differences exist between cells adhering to different types of substrata. This may be due to the inhibitory effect of the chemicals associated with the substrata, affecting growth and division of the cell. Many microbial species are pleiomorphic and can be significantly affected by changes in environmental conditions. Novitsky and Morita (1976, 1977) demonstrated that a pure culture of a marine Vibrio spp possessed different morphological forms depending upon the nutrient status of the environment. When grown under a nutrient limitation, the cell reduced in size from 1.0μm long to a dwarf form 0.5μm long. Restoring the cells to a sufficient nutrient concentration led to them regaining their normal size and activity. Very small bacteria tend to be the primary colonisers of surfaces immersed in marine habitats (Marshall et al., 1971b) but appear to be displaced on the surface by normal sized bacteria after 12 hours or more (Marshall, 1980). Dawson et al.,(1981) proposed that the small bacteria are in fact starved dwarf forms that adhere in a tactic for survival. The larger forms that are subsequently observed are in many cases the same cell type that are able to grow normally due to increased nutrient levels. Evidence supporting this has been presented by Kjelleberg et al.,(1982). Individual small starved cells at a nutrient enriched solid/water interface exhibited a rapid but transient increase in cell volume.

Marshall & Fletcher (1982a) proposed that in most aquatic habitats two major groups of bacteria are recognized; the oligotrophs, capable of growth in low nutrient environments, and the copiotrophs, requiring relatively high levels of nutrients for growth. When a surface is immersed in an aqueous environment it will act as a source of nutrient concentration. The copiotrophic bacteria will be at a selective advantage, colonizing the surface first followed by the oligotrophic species. This successional development has been reported frequently (Zobell, 1943; Marshall et al., 1971b;

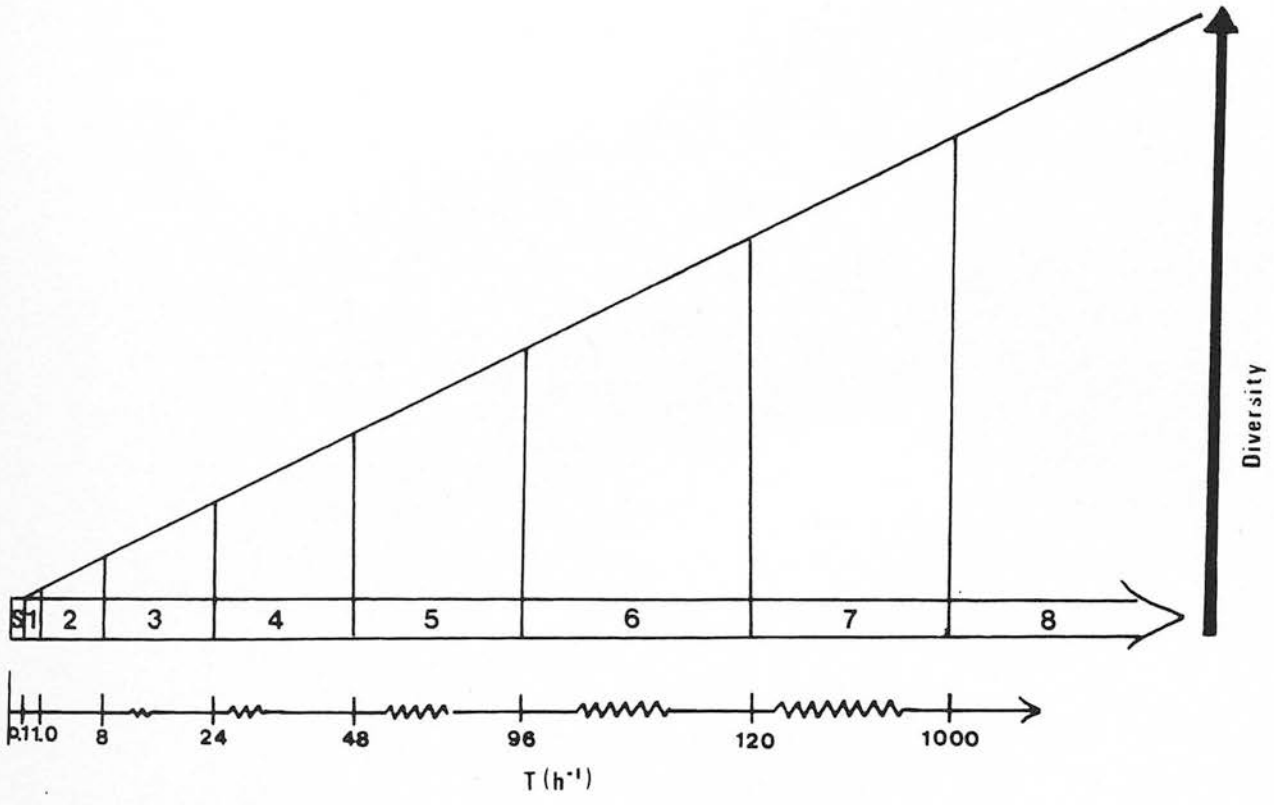
Corpe, 1972). A rapid accumulation of Pseudomonad species was initially observed on a glass slide submerged in seawater, replaced by Caulobacter, Hyphomicrobium and Saprospira species after 48-72 hours (Corpe, 1972). After 7 days submersion attached cell numbers exceeded 2 x 10^6 cells cm^{-1}. However, only 10-15% of the organisms were gram positive. The composition of the microbial film becomes increasingly complex with time and varies with the nature of the initial surface (Gerchakov et al., 1977; Dempsey, 1981a+b). Marszalek et al.,(1979) made visual comparisons of the fouling communities of glass, stainless steel and brass surfaces. Glass and stainless steel showed similar fouling characteristics, a two tier microfouling layer initially colonized by short rod shaped bacteria closely associated with adhesive material. Results from the brass were very different. Build up was slower, though the area covered with slime increased without any obvious increase in cell numbers.

Limiting nutrients by continuous culture can markedly affect the microbial population obtained. Wardell et al.,(1980) discovered that under carbon limitation Aeromonas species was dominant, accounting for at least 90% of the total attached population. With nitrogen limitation Aeromonas was again present, as were Pseudomonas sp, Flavobacterium sp and Vibrio species. When using aluminium foil as the substratum in nitrogen limitation, a film of polysaccharide material could be observed.

The inter-relationships between different microbial special can be complicated and difficult to evaluate. Sieburth (1967) observed that an inverse relationship existed between Arthrobacter sp and Pseudomonad sp isolated from Atlantic seawater. The Pseudomonad produced an inhibitor of low molecular weight and an agglutinin which reacted with the Arthrobacter cells. The inhibitor in sufficient concentration could inhibit Arthrobacter development.

A complicated situation exists in the microfouling of surfaces though the process can be generalized (fig.10). Different surfaces are fouled at different rates, some being more selective for certain organisms than others (Dempsey, 1981b). The production rate of attached extracellular polysaccharide increases during film development. Attached polymer increases nonlinearly, whereas the biomass increases linearly (Bryers & Characklis, 1982).

FIGURE 10: Generalized Diagram of Successional Development



Key:

S Inert Surface

1 Organic Conditioning Film

2 Short rods, dwarf forms - Copiotrophic Chemo-organotrophs

3 Rods, cocci, spiral forms

4 Stalked forms - Oligotrophic Chemo-organotrophs

5 Fungi

6 Diatoms, microalgae

7 Protozoa

8 Invertebrates

8.2 Algae

Normally microalgae are not primary colonizers of surfaces (Marshall et al., 1971b ; Corpe, 1972) but appear as a later stage in the successional development of surfaces (fig.10). Attachment mechanisms are generally by the secretion of adhesive mucilagenous material, although the rooting algae Rhizophyte produces a holdfast structure to provide an anchor to the substratum (Corpe, 1980). Among the sessile diatoms several attachment systems of varying morphology have been described (Chamberlain, 1975). These include forms with an adhesive pad, a mucous capsule, a flattened tubular sheath surrounding colonies and mucilagenous branching stalks. The algae Chlorella vulgaris secretes polymeric material containing protein and carbohydrate which enhances adhesion (Tosteson & Corpe, 1975). It was thought that the increased adhesion was possibly a secondary effect resulting from aggregation of the algal cells induced by the polymer and subsequent secretion of further algal adhesive (Sutherland, 1983a). Glycoprotein adhesive material is also produced by other algal groups (Evans & Christie, 1970) and in some cases the site of formation is believed to be the golgi apparatus (Baker & Evans, 1973).

8.3 Fungi

Fungi can resist removal by rinsing suggesting either that fungal cells are protected by irregularities or surface structures characteristic of the substratum, or that attachment is facilitated by some property of the fungus (Corpe, 1980). Many filamentous fungi and yeasts secrete extracellular polysaccharides with adhesive qualities (Martin & Adams, 1956). The sugar components from the EPS fractions of the Mucor and Rhizopus sp sampled contained glucuronic acid, fucose, galactose, mannose and small amounts of glucose. Filamentous fungi are found widely distributed in soil, water and on surfaces of almost every description. The colonization of wooden surfaces produced a surprising result, there being a lack of fungi on the surface after 12 weeks (Cundell & Mitchell, 1977). In an examination of "sewage fungus" (Curtis & Curds, 1971) it was noted that the most common organisms to occur behind Sphaerotilus natans and zoogloeal bacterial forms were the fungi Leptomitus lacteus and Geotrichum candidum. Adhesive mechanisms

can range from the discharged contents of peripheral vesicles acting as an adhesion (Sing & Bartnicki-Garcia, 1975) to the production of an EPS which can flocculate clay (Zajic & LeDuy, 1973).

SECTION 9 Advantages of Adhesion to the Cell

Surfaces profoundly affect microbial activity and growth, providing an area where growth and survival can take place (Marshall, 1976). A beneficial effect of a solid surface is usually evident in only very low nutrient concentrations, ie. less than $10mgL^{-1}$ (Hattori & Hattori, 1976). In an aqueous environment, freely suspended cells are assumed to exist under conditions of nutrient deprivation (Jannasch, 1969). It has been well proven that the addition of solid surfaces under these conditions could facilitate bacterial growth (Zobell, 1943; Hendricks, 1974), the surfaces concentrating nutrients in an otherwise nutrient depleted environment. Zobell (1943) calculated that 2-27% of the total organic content of seawater adsorbed onto glass, when given $2\text{-}200cm^{2}ml^{-1}$ of additional surface area. Free floating bacteria exhibited a lower amino acid turnover overall than attached bacteria (Bell & Albright, 1982) indicating that attached bacteria have an ecological advantage over the planktonic population (Paerl, 1980). Bacteria are found at interfaces because of a nutrient accumulation from the bulk aqueous phase (Kjelleberg et al., 1982).

Attached organisms have two clear advantages in flowing water; 1) they do not need to expend much energy searching for food, as water with fresh nutrients flows over them removing waste products at the same time. 2) In fast flowing waters washout would occur without anchorage to a surface (Fletcher & Marshall, 1982a).

Attachment to a substrate is beneficial for assimilatory purposes. The production of enzymes is related to the attachment of a cell to the substrate (Minato & Suto, 1976), the enzyme being put to a more efficient use by being retained within the adhesive matrix rather than diffusing into the surrounding medium. Cytophaga sp which are capable of decomposing solid substrates such as cellulose, chitin, agar or alginate in aquatic habitats benefit by this process (Fletcher & Marshall, 1982a).

Other advantages of attachment include cross feeding, co-metabolism, interspecies hydrogen transfer and interspecies organism proton transfer (Wardell et al., 1980). An enhancement of the chemiosmotic theory has also been proposed (Ellwood et al., 1982).

There is a limit to the extent to which EPS can promote nutrient accessibility. In biofilms exceeding 70μm in thickness, the diffusion of nutrients to underlying layers becomes restricted. This can also lead to the diffusion rates of gases and toxic by-products being impeded (Geesey, 1982). A further disadvantage of bacterial adhesion is that the cells can become prone to grazing by other organisms, namely, Protozoa and Mytilus sp (fig.11).

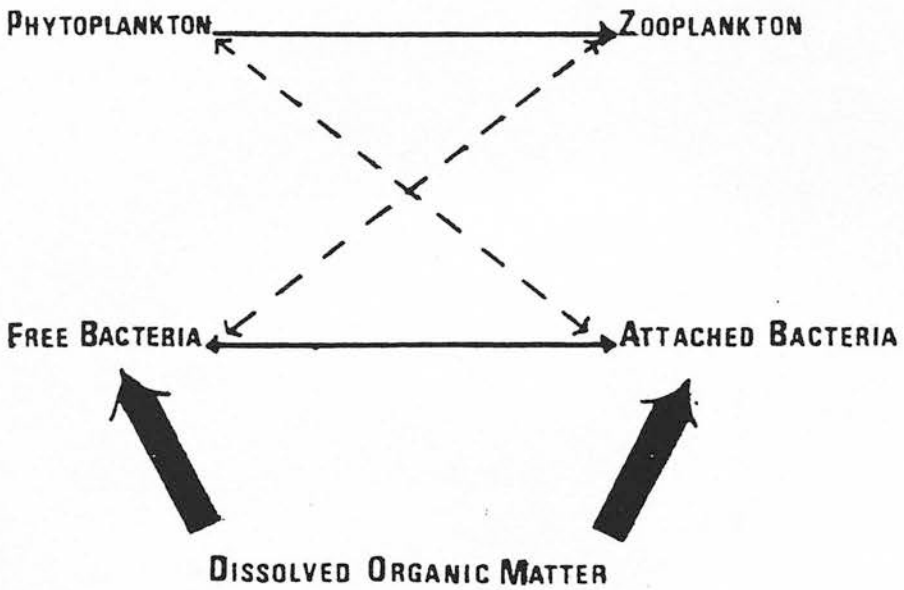


FIGURE 11: The main relationships of bacteria in the sea with other components of the ecosystem. Adapted from van ES and Meyer-Reel (1982).

SECTION 10 Economic Consideration of Bacterial Adhesion

10.1 Advantages

Associations between bacteria and surfaces have been observed in many environments (Berkeley et al., 1980), the majority causing a detrimental effect to the surface in question (Section 10.2). There is however, a more positive aspect to surface growth. The performance of a trickling filter in the sewage process is wholly dependent upon the slime layer. Micro-organisms attach to the packing material of a trickling filter forming a reactive surface for the adsorption and breakdown of organic wastes (Characklis, 1973). The EPS found in the mucilage and slime layers of algae and bacteria isolated from various natural sources have exhibited heavy metal binding capacities (Geesey, 1982). Similarly, pesticides such as aldrin are bound by suspended flocculant particles, the net effect being the removal of the pollutant from solution. Other advantages conferred by bacterial adhesion include special fermentation processes such as vinegar production from ethanol (Bryers & Characklis, 1982) and the development of tissue culture reactors (Powell & Slater, 1982). The oral administration of particulate material has been shown to inactivate adsorbed pathogens, kaolin removing organisms not normally found in the intestinal tract (Gunnison & Marshall, 1937).

10.2 Disadvantages

The disadvantages and problems caused by bacterial adhesion far outweigh the advantages. Biological fouling of man-made structures such as dams, platforms and ship hulls can lead to severe corrosion and increased operating costs. To repaint on an average 250,000 tonne dead weight supertanker costs between £40,000 and £50,000 on an annual basis (Dempsey, 1981b). The biological fouling of carbon columns is a constant problem in wastewater and water treatment operations. Anaerobic conditions occur resulting in the production of sulphide and other undesirable products (Characklis, 1973).

"Bulking" in sewage treatment occurs when the activated sludge will not settle properly, compacting poorly. The organisms Sphaerotilus natans and the closely related Beggiatoa spp are the principle organisms causing this problem (Pipes, 1967). S.natans

also causes problems of obstruction and corrosion in pipes, interfering with commercial fishing and waste disposal (Dondero, 1961). The blocking of pipes increases the frictional resistance in flow systems, leading to a greater head loss. Marszalek et al.,(1979) calculated that a fouling film of organisms 55 μm thick would lead to an acceptable loss in heat transfer in heat exchangers. Ecologically, the formation of slime blooms (Curtis & Curds, 1971) is both unsightly and can lead to serious health risks. A cross-sectional survey of the Danube river showed there to be 64 tons wet weight per day of drifting Sphaerotilus and associated organisms (Dondero, 1961).

10.3 Prevention

Because of the problems discussed above, it is desirable that some means of control be devised to help prevent microfouling occuring. In some cases the use of biocides has eliminated growth of fouling micro-organisms in many industrial processes (Geesey, 1982). Hypochlorite has been the most feasible means of control for large scale operations, as it reacts with the EPS causing a depolymerization of the matrix and denaturation of proteins. Other considerations have included polysaccharases and proteolytic enzymes (Dalielsson et al., 1977). Use of the nucleoside 9-β-D arabinofuranosyladenine (ara-A) at low concentrations has prevented the attached growth of S.natans (Takiguchi et al., 1978 ; Yoshikawa and Takiguchi, 1979). Attached growth was inhibited by concentrations between 0.5 and 1.0μgml^{-1} ara-A if applied to the cells before filaments formed. The nucleoside appears to be a selective inhibitor of attachments as it fails to affect the growth of other organisms tested at concentrations greater than 1mgml^{-1}. An elegant study by Dempsey (198la,b) used SEM to study the effect of painted surfaces containing toxic agents upon bacterial adhesion. A great diversity of bacterial species with varying anchorage mechanisms was observed. Non-toxic paints contained organisms adhering through strands of polymer, whereas organisms on copper based paint were covered in sheets of slime. Little polymeric material was observed initially on paints containing triphenyl tin fluoride, but prolonged incubation did lead to further accumulation. Anti-fouling paints have the problem though that they are only effective for 1-3 years before repainting

is needed, and the concentrations used are toxic to other life forms. The use of organic acids has been shown to have an inhibitory effect (Chet et al., 1975). Both tannic and benzoic acid were found to reduce slime formation on surfaces from $3.45 mgcm^{-2}$ after 40 days to $0.2 mgcm^{-2}$, the reduction being attributed to negative chemotaxis.

A three point plan proposed by Costerton et al.,(1978) to inhibit attachment would appear to have little value in the natural environment where the organisms concerned are very ofter unidentified. This highlights the problem of microbial fouling, as very little as yet is known about the substratum colonization and the strength of adhesion and successional development on surfaces (Fletcher & Marshall, 1982a).

CHAPTER TWO: MATERIALS and METHODS

Materials and Methods

Bacterial Strains

The bacterial strains used in this study were isolated from surfaces immersed in a fast flowing river. Both synthetic and natural surfaces were used. Stock cultures were maintained on a semi-synthetic medium 1/10 Yeast Extract (1/10 YE) slope at 4°C. To ensure strain stability all isolates were lyophilized.

The Saccharomyces cerevisiae strain used was provided from a laboratory culture, maintained on nutrient agar slopes at room temperature.

Mutants derived from the parental strains were maintained under similar conditions.

Growth of Bacteria

Liquid cultures were routinely grown in YE media (Table 4), in either 100ml volumes in 250ml flasks, or 1-Litre volumes contained in 2-Litre Erlenmeyer flasks at 120rpm. Plate growth was performed on 1/10 YE media solidified with 2% (w/v) agar (Table 5). Both liquid and solid media were supplemented with 1% (w/v) glucose and grown at 30°C unless otherwise stated.

Extracellular Polysaccharide Production

a) Preparation

Two methods were used, 1) Cultures were grown on 1/10 YE agar supplemented with 1% (w/v) glucose. 600ml aliquots of medium were contained in shallow enamel trays (35 x 25cm). Cells were removed after 96h growth with a glass slide and resuspended in physiological saline (0.85% NaCl w/v) containing five drops of formalin per 100ml, using a blender. The bacteria were removed by centrifugation at 30,000g for 30 min. (Sorval RC-5B refrigerated Superspeed Centrifuge, DuPont Instruments, Delaware, USA). Exopolysaccharide was precipitated from the supernatant by addition to 2 vols. of cold acetone.

2) Liquid cultures, grown for 96h were centrifuged to remove cells, formalin added to the supernate, and EPS precipitated by addition to acetone.

b) Purification

Precipitated polymer was resuspended in distilled water and reduced in volume by rotary evaporation. Purification was

TABLE 3: Yeast Extract (YE) Salts

Na_2HPO_4	1.0g
KH_2PO_4	0.3g
K_2SO_4	0.1g
NaCl	0.1g
$MgSO_4 7H_2O$	0.02g
$CaCl_2$	0.1ml of a 1% solution
$FeSO_4$	0.01ml of a 1% solution
Distilled Water	1 Litre

TABLE 4: YE Media

Glucose	10.0g auto claved separately
Casamino Acids	1.0g
Yeast Extract	1.0g
YE Salts	1 Litre
pH	7.2

TABLE 5: 1/10 YE Media

Glucose	10.0g auto claved separately
Casamino Acids	0.1g
Yeast Estract	0.1g
YE Salts	1 Litre
pH	7.2

TABLE 6: Phosate Buffered Saline (PBS)

NaCl	8.0g
Na_2HPO_4	1.15g
KCl	0.2g
KH_2PO_4	0.2g
Distilled Water	1 Litre
pH	7.2

achieved by ultra centrifuging the polymer (200 000g for 3h; MSE Prepspin 65, Sussex, England) and dialysing the supernatant against running tap water for 48h and distilled water for 24h. When all traces of acetone had been removed, the polymer was frozen and freeze-dried.

Mutagenesis

UV irradiation

Washed, logarithmic phase cells were resuspended in 10ml citrate buffer (Cruickshank, 1965) and transferred aseptically to a sterile glass petri-dish. This was placed below a 260nm UV source, the lid removed, and the cells irradiated for 5 min. 100ul samples were used to inoculate 9.9ml volumes of YE media, grown overnight, and serially diluted on to 1/10YE agar plates.

Gamma irradiation

Irradiation of washed logarithmic cells was carried out using a ^{60}Cobalt source with 0.5-3min. exposure. Samples were diluted and plated directly.

Uranyl Salts

The method of Ark (1951) was employed. Cells were grown overnight in 9ml quantities of nutrient broth and 1ml of mutagen added ($20gL^{-1}$). Samples were removed every three days for a total of twelve days, the culture being diluted to 10^{-6} and 0.1ml aliquots plated onto 1/10 YE agar.

N-Methyl-N-Nitroso-N-Nitroguanadine (NTG).

Washed, logarithmic phase cells were resuspended in 10ml phosphate buffer pH7.0 containing 30-60μgml^{-1}NTG and incubated for 30 min. Cells were pelleted (30 000g, 20 min), washed, and resuspended in YE medium. Following overnight incubation, cells were serially diluted and plated out.

Minimum Inhibitory Concentration

Doubling dilutions were carried out in sterile $\frac{1}{4}$ oz. vials containing 1ml final volume of YE broth and filter sterilized antibiotic. 1:100 dilutions of overnight culture was added aseptically and incubated for 16h. The lowest dilution at which growth was inhibited was taken as being the minimum inhibitory concentration.

The above method was also used to determine the sensitivity of stock cultures to 9-β-D-arabinofuranosyladenine (Ara-A).

Assay of Growth Curve Exopolysaccharide Production

a) EPS

2ml samples of culture were treated with one drop of formalin and dialysed exhaustively against running tap water (120h) and distilled water (24h). Cells were removed by centrifugation (9 000g, 10min) and the amount of exopolysaccharide present determined by the phenol-sulphuric acid assay (Dubois et al., 1956).

b) Residual Glucose

1ml samples of culture were diluted 1:2 with distilled water, mixed thoroughly, and the cells pelleted (9 000g, 10min). Residual glucose was determined on 1:100 dilutions of the cell free culture using the glucose oxidase method (Boehringer Corporation, London).

Hydrolysis of Exopolysaccharides

Polysaccharides were hydrolysed by heating 5mg in 600µl 1N Trifluoroacetic acid for 16h in sealed glass ampoules, 100°C. The hydrolysate was neutralized by repeated washing with distilled water and evaporating under pressure (X3).

Chromatography of Polysaccharides

Concentrated hydrolysate was re-dissolved to a final volume of 100µl. 5µl was applied to Whatman No.1 paper and separated by descending chromatography. The chromatograms were irrigated by either solvent A or solvent B, running for 40h.

Solvent A	Butanol/Pyridine/Water, (6:4:3)
Solvent B	Ethylacetate/Acetic Acid/Formic Acid/ Water, (18:3:1:4)

Standards were run as 2µl amounts of 0.1M solutions.

Detection of Chromatographic Material

Sugars were detected by the alkaline silver nitrate method (Trevelyan et al., 1950).

Measurement of Polysaccharide Viscosity

2ml samples of 0.5% (w/v) EPS samples were measured at increasing shear rates using a Brookfield Digital cone plate viscometer.

Exopolysaccharide Analysis by Gel-Filtration

EPS was analyzed on a sephacryl superfine 500 column (30 x 1cm, Pharmacia, Uppsala, Sweden) by eluting 500µl of a 2mgml^{-1} solution with distilled water. 0.75ml fractions were assayed for total un-

hydrolyzed carbohydrate (Dubois *et al.*, 1956) using D-glucose as a standard.

Haemagglutination Assay for Fimbriae

The method of Tiffin (1982) was employed. Bacterial cells were grown statically overnight, washed and resuspended in saline (X2) and pelleted. Fresh blood was washed in saline to make a 3% (v/v) suspension. The bacterial cells and blood suspension were mixed in a spotting tray and left overnight at $7^{o}C$. A lab strain of *S.cerevisiae* was used as a positive control.

Isolation of Outer Membrane Proteins

1 Growth of Cells

2 x 100ml samples of cells were grown overnight, then added to 1 Litre of fresh medium (20% v/v inoculum) and grown until an E_{600} of 0.8 units was reached. Cells were harvested (27 000g, 30 min), resuspended in 15% (w/v) glycerol and frozen as 20ml aliquots at -80°C.

2 Isolation of Membraneous Material

a) The procedure of Osborn et al.,(1972) was adopted: Cells pelleted from 1L of culture were resuspended in 100ml of cold 0.75M Sucrose, 10m M Tris-HCl buffer, pH 7.8. Lysozyme was added immediately (5ml of a $2mgml^{-1}$soln) and incubated on ice for 2 min. Two volumes of cold 1.5mM EDTA (sodium salt) pH 7.5 was added over a 15 minute period, taking care to add the EDTA below the cell suspension surface which was being constantly swirled. Sphaeroplasting was checked by phase contract microscopy. Unlysed cells and debris were removed by centrifugation (5 000g, 10 min) and the resulting supernate spun at 50 000g (45 min) to sediment the sphaeroplasts. The pellet was resuspended in 18ml of distilled water and sonicated (30 second exposures) at a setting of 7 microns in a MSE 100W Ultrasonicator (60 c/s) until the absorbance of the sphaeroplast suspension had decreased to approximately 10% of its original value. The membranes were pelleted (100 000g, 45 min) and subsequently washed in distilled water.

All steps were performed on ice or at 4°C.

b) Phosphate-buffered saline treatment (PBS) (Table 6). Cells harvested as before, were washed and re-spun in PBS (35 000g, 25 min). The pellet was resuspended in 18ml of distilled water and sonicated as above. Cell debris was removed by centrifuging at 5 000g, 10 min. the membranes being pelleted by high speed centrifugation (50 000g, 30 min).

3 Separation of Inner and Outer Membranes

a) Sarkosyl separation for Osborn prepared membranes: The membranes were resuspended in 10ml of 1% (w/v) sarkosyl and incubated in a shaking water bath at room temperature for 20 min. The suspension was subsequently centrifuged (150 000g, 45 min), the pellet obtained washed once in distilled water, frozen, and freezed dried. This was the outer membrane, the sarkosyl soluble fraction being the inner membrane.

b) Sarkosyl separation for PBS prepared membranes: Membranes were resuspended in 5ml of 20% sarkosyl (w/v) and incubated as above for 30 min. The outer membrane fraction was pelleted (150 000g, 30 min) and washed in PBS. As before, the pellet was frozen and lyophilized.

Polyacrylamide Gel Electrophoresis of Membrane Proteins

1 Method

Outer membrane material prepared as described above, was solubilized in an equal volume of double strength sample buffer at 100°C (10 min), cooled, and 25µl loaded onto a gel. Acrylamide gels were prepared in cassettes (18x16x0.1cm) comprising chromic acid cleaned glass plates and perspex spacers sealed with soft paraffin wax. A 5% stacking gel (pH 6.8) was layered upon the lower resolving gel. Electrophoresis was performed in equipment supplied by Raven Scientific Ltd., (Haverhill, Suffolk). An initial constant current of 15mA was used unitl the dye front entered the resolving gel and was subsequently run at 20mA until the front was 1cm above the base of the gel. A constant current of 2-3mA was used for gels run overnight.

Protein was stained using either PAGE blue 83 with subsequent destaining, or by silver staining. Alcian blue was used to stain polysaccharide material.

2 Materials

30% stock acrylamide, comprising 29.2% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide filtered through glass wool and stored at 4°C in a dark bottle.

Resolving gel: Run as either a linear gradient, or as a non gradient gel, composed of stock acrylamide; 1.5M Tris-HCl (pH 8.8) 0.4% SDS, 0.25 vol; distilled water. In non-gradient gels, 0.00125 vol of 10% (w/v) ammonium persulphate catalyst was added after degassing the acrylamide solutions, followed by 0.00235 vol. of TEMED accelerator. In a gradient gel, the volumes used were similar for the accelerator, but were 0.0019 vol. and 0.0028 vol. for the catalyst in low and high concentration acrylamide, respectively. Oxygen was excluded from the poured gel by applying a layer of saturated butyl alcohol until polymerization occurred.

Stacking gel: comprised of 0.16 vol stock acrylamide; 0.25 vol. 1.5M Tris-HCl/pH 6.8) 0.4% SDS; 0.59 vol. distilled water; 0.0028 vol. catalyst and 0.0016 vol. accelerator.

Sample buffer: Final concentrations were:-

0.01M Tris-HCl (pH 6.8)

1% SDS

0.1% β-mercaptoethanol

10% Glycerol

0.005% Bromophenol blue

Samples containing approximately $1.0\mu g\mu l^{-1}$ protein were applied to the gel.

Running buffer:	Tris-base	$3.0gmL^{-1}$
	Glycine	$14.4gmL^{-1}$
	SDS	$1.0gmL^{-1}$
PAGE blue stain:	Page Blue 83	1.25gm
	Methanol	227ml
	Glacial Acetic Acid	46ml
	Distilled Water to	500ml - stir and filter

Stain immersed gel for a minimum of 4h with gentle agitation.

Destain:	Methanol	50ml
	Glacial Acetic Acid	75ml
	Distilled Water to	1000ml Destain changed regularly over saveral days until background of gel was cleared.
Silver stain:	Biorad labs., bulletin 1089	

Calculation of Molecular Weights

Protein standards were applied to the gel at concentrations of $1\mu g\mu l^{-1}$ in sample buffer. To take account of swelling gels during staining and destaining, the following calculation was adopted:-

$$Rf = \frac{\text{Distance of Protein Migration}}{\text{Length of gel after destaining}} \times \frac{\text{Length of Gel Before Destaining}}{\text{Distance of dye migration}}$$

A plot of $\log_{10}$ molecular weight of the standards against Rf value results in a standard curve.

Polyacrylamide Gel Densitometry

1cm wide longitudinal tracks were cut from thoroughly destained gels. Each slice was washed gently with distilled water, placed in a cuvette and scanned at 280nm ($25mm\ min^{-1}$) in a Joyce-Loeble Scan 400 (Joyce-Loeble, Gateshead, England).

In vitro Attachment Studies

1 Visualization of Cells and Associated Polymer

Chemically clean, sterile glass slides (75x25mm) were suspended in a 1 Litre culture in a 2 Litre Erlenmeyer flask, incubated at 30° on a reciprocating shaker (120rpm). Sampling was performed at regular intervals. On removal, the surface of the slide was covered with 10mM cetylpyridinium chloride and air dried for 20-30 min. Following gentle heat fixing, the cooled slides were stained for 15 min. with a 1:2 mixture of saturated aqueous congo red solution and 10% (v/v) Tween 80 solution. After careful rinsing, the slides were stained with 10% (v/v) carbol fuchsin for 2 min., rinsed, and allowed to air dry at 37°C.

2 Estimation of Adsorbed Carbohydrate Material

Chemically clean glass coverslips (18x18mm) were suspended in the cultures and removed at regular intervals. Coverslips were also suspended in cell free media to act as a control. Upon removal, the coverslips were rinsed (x3) in distilled water, crushed, and assayed for adsorbed carbohydrate material by the phenol-sulphuric acid assay (Dubois *et al.*, 1956). D-glucose was used as standard.

3 Estimation of Attached Cell Numbers

Cells were harvested (27 000g, 30 min), washed in YE salts (Table 3), and resuspended to an appropriate concentration in YE salts. Chemically clean glass slides (35x25mm) were submerged for varying periods of time. Upon removal, the slides were rinsed with sterile YE salts and swabbed. A dry sterile cotton wool swab was used first followed by a swab moistened in YE salts. Both were vortexed in 10ml sterile YE salts for 20 seconds and serially diluted before plating.

Photography

A leitz Orthoplan Microscope (100x, oil immersion) with camera attachment (Wetzlar, FRG) was used for all photographs with Kodak Ektachrome 160 film.

Scanning Electron Microscopy

1 Cell Monolayer

Fixation and visualization were carried out as a standard protocol (Robards, 1978; Glauert, 1975). Chemically clean glass coverslips (13mm diam.) were suspended in the bacterial culture, removed at regular intervals and rinsed in sterile YE salts. Fixation for 2h in 0.1M cacodylate buffer pH 7.2 containing 2.5% (v/v) glutaraldehyde and 3.0% (w/v) tannic acid was followed by two 10 min. washes in distilled water. Further fixation in 2% (v/v) osmium tetroxide in 0.1M cacodylate buffer for 2h was again followed by two 10 min. distilled water washes. Dehydration occurred through an acetone series, (10%, 20%, 40%, 60%, 80%, 90%, 100%, 100%, 100%) 5 min. each. The samples were subjected to critical point drying (Polaron critical point drier, Watford, England) using acetone, and CO_2 as the transitional fluid, attached to stubs using double sided tape, and sputter coated with gold (2 min. at 20mA deposition) (Emscope SC500 modular sputter coater, Kent, England).

2 Unattached Cells

Chemically clean coverslips were pre-coated with 20µl of 0.1% (w/v) polylysine solution and allowed to dry. Cell suspensions were added to an equal volume of buffered 5% (v/v) glutaraldehyde and pelleted in a microfuge tube. Upon removal of the supernate, samples of the pelleted cells were smeared across the pre-coated coverslip and subsequently fixed as above.

3 Observation

An accelerating voltage of 40kV was used to observe the specimens in a Cambridge Stereoscan 250 scanning electron microscope (Cambridge, England).

Nikon 35mm film was used for photography.

Continuous Culture

All continuous culture experiments were performed using a New Brunswick Bioflo C-30 chemostat (fig.12). The working volume of the fermentation vessel was 310ml. Samples were taken direct from the culture vessel, discarding the first sample containing residue from the sample line dead space each time. Medium was fed into the culture vessel at a controlled rate using a LKB peristaltic pump.

Inoculation was from a batch culture, via a sterile syringe inserted through a rubber membrane over the inoculation port. Once inoculated, the culture was grown for 8h at a low dilution rate ($D=0.010h^{-1}$) to allow the cells to adapt to the chemostat conditions. Samples were taken regularly until steady state values were achieved for each D value; at this stage, material was harvested for further analysis.

Culture Analysis

The following parameters were measured:-

i	E_{600} Culture
ii	Cell Count
iii	pH Culture
iv	EPS Dry Weight
v	Cell Dry Weight
vi	Culture Residual Glucose
vii	Total Carbohydrate Produced
viii	Viscosity of Culture
ix	Polysaccharide Viscosity

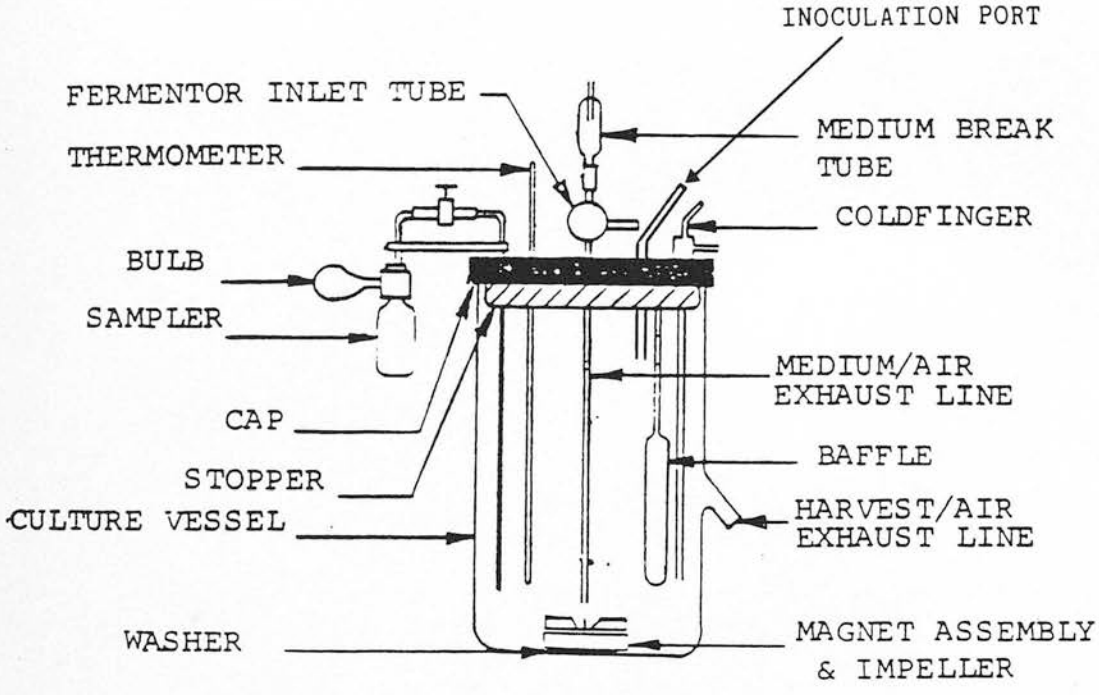


FIGURE 12: Fermentor assembly components.

Affinity Chromatography

Outer membrane material was analyzed for specific cell surface receptors by application to immobilized sugar (Pierce Chemical Company, Illinois, USA) affinity chromatography columns (5 x 0.5cm). 750µl of sample was initially eluted with 20mM phosphate buffer (Cruickshank, 1965) containing 0.1M NaCl (pH 7.0) until unattached protein washed through. Phosphate buffer (20mM) containing 0.1M NaCl and 0.3M of the respective sugar (pH 7.0) was used to elute specifically adsorbed protein. Remaining protein was removed and the column washed with a 20mM phosphate, 2M NaCl buffer (pH 7.0). All buffers contained 0.02% (w/v) NaN_3. Fraction size was 850µl, protein measured by absorbance at 280nm.

Fine Chemicals and Biochemicals

All chemicals and biochemicals were of the purest grades available, purchased from either BDH Chemicals Limited, Poole, England, or the Sigma Chemical Company Limited, Kingston Upon Thames, England, unless otherwise stated.

Analytical Techniques

All analytical methods were performed as micromodifications of original assay procedures.

Protein was measured by the method of Lowry *et al.*, (1951) or Markwell *et al.*, (1978).

Total carbohydrate was determined by the phenol-sulphuric acid reaction of Dubois *et al.*, (1956).

Pyruvate was estimated by the dinitrophenylhydrazine method of Sloneker and Orentas (1962).

Acetate was measured by the Hestrin (1949) technique.

Glucose was determined using glucose oxidase (Boehringer Mannheim, GmbH, W. Germany).

CHAPTER THREE: RESULTS and DISCUSSION

SECTION 1 Isolation of Adherent Freshwater Bacteria

1.1 Introduction

Many bacteria are able to attach to solid surfaces in almost any natural environment. Attached populations are particularly significant in low nutrient waters, adhesion offering some nutritional advantage to the organisms. In most environments there appears to be a distribution between the attached bacteria and those which are free and unassociated with suspended matter. Geesey and Costerton (1979) showed by epifluorescence microscopy that 76% of the bacterial population in one sample of a North American river were free living, the remaining 24% being associated with the surfaces of diatoms and amorphous material. Similarly, Bell and Albright (1982) reported that in the open ocean, bacteria are mainly unattached and free floating.

Attached bacteria found in fast flowing freshwater frequently produce copious amounts of exopolysaccharide (Characklis, 1973; Geesey et al., 1977). Yet, despite the interest and attention paid to the adhesion process from Zobells (1943) initial study, little is known about the organisms that adhere and their relationship to the total aquatic population. Characterization of these bacteria has been largely neglected.

1.2 River Characterization

A clean, fast flowing stream to the south-west of Edinburgh was used to isolate adherent bacteria for this study. The surrounding soil type was impervious clay loam, retaining 95% surface water, the bed rock being sandstone in a lower shield series. Average temperature and pH measurements were $9^{o}C$ and 7.8, respectively. Atomic absorption spectroscopy was used to measure the concentration of five major inorganic ions (Table 7).

Ion	Concentration
Ca^{2+}	$37.2\mu gml^{-1}$
PO_4^{3-}	$80.6\mu gL^{-1}$
Mg^{2+}	$35.6\mu gml^{-1}$
Na^{+}	$7.9\mu gml^{-1}$
K^{+}	$67.6\mu gL^{-1}$

Table 7: Ionic Composition of River Water Sample.

1.3 Bacterial Isolation

Chemically clean glass slides were suspended in the river (below the air water interface) and removed after 24h. Bacteria were isolated in pure cultures by standard procedures, grown on 1/10 YE agar. Strains were selected for further work on the basis of a mucoid colonial appearance.

1.4 Bacterial Characterization

1.4.1 Biochemical Tests

Elementary biochemical tests were performed on 15 of the freshwater isolates showing varying degrees of polysaccharide production. These included range of growth temperature, catalase production amylase production, citrate utilization and anaerobiosis. All methods employed were standard techniques described in Cruickshank (1965). The results (Table 8) indicate that most isolates are capable of growth between 7°c and 37°c, produce either catalase or oxidase (strain R4d produces both, S61 neither) and virtually all utilize both urea and citrate (strain R4c lacks urease). Only three of the isolates are facultative anaerobes (S61, R3a and R3c). The methyl red and Voges Proskeur tests indicate that strains SIc, S4c and 2S3b may possibly be members of the Enterobacteriaceae. More detailed information is needed for any further identification.

1.4.2 Carbohydrate Metabolism

Carbohydrate metabolism was assayed for a variety of sugars using the method of Hugh and Leifson (1935). Aerobic and anaerobic breakdown of carbohydrate material can be distinguished on the basis of acid production. Solid media (tubed) containing the carbohydrate and a pH indicator (bromothymol blue) produces acid only at the surface when oxidative breakdown occurs; acid throughout the tube indicates fermentation. 1% solutions of pentoses, hexoses, disaccharides, polysaccharides, glycosides and sugar alcohols were tested. The results (Table 9) show that strains S4c and R3c ferment all the available sugars, R3c within 48h producing notable amounts of gas. This further suggests strain S4c being a member of the Enterobacteriaceae. Strains S61, RIa, RIb and 2S3b show only an oxidative breakdown on selected substrates, 2S3b being able to oxidise only the pentose and hexoses.

Strain	Gram Stain	Cell Length	Growth °C 3	7	12	17	25	30	37	44	Catalase	Oxidase	Methyl Red	Voges Proskeur	Urease	Citrate	Starch	Nitrate	Gelatin	Anserobic
S61	-	3.4µm	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-	+
S1c	-	1.4µm	-	-/+	+	+	+	+	+	-	+	-	-	+	+	+	-	+	-	-
S4c	+	6.8µm	-	-	+	+	+	+	+	-	+	-	+	-	+	+	-	+	-	-
R1a	-	2.7µm	-	-	+	+	+	+	-	-	+	-	-	-	+	+	+	-	-/+	-
R1b	-	2.0µm	-	-/+	+	+	+	+	-	-	+	-	-	-	+	+	-	-	+	-
R3a	-	1.4µm	-/+	+	+	+	+	+	+	-/+	+	-	-	-	+	+	-	+	+	+
R3b	-	3.4µm	+	+	+	+	+	+	+	-	-	+	-	-	+	+	-	-	-	-
R3c	-	1.4µm	-/+	+	+	+	+	+	+	-/+	+	-	-	-	-/+	+	-	+	+	+
R4b	-	2.7µm	-	-	+	+	+	+	+	+	+	-	-	-	+	+	-	-	-	-
R4c	-	6.8µm	+	+	+	+	+	+	-/+	-/+	-	+	-	-	-	+	-	-	-	-
R4d	-	4.1µm	-	-/+	+	+	+	+	-	-	+	+	-	-	+	+	+	-	-/+	-
2S3b	-	2.7µm	-/+	+	+	+	+	+	-	-	+	-	+	-	+	+	+	-	-	-
2S4a	-	2.7µm	-	+	+	+	+	-	-	-	+	-	-	-	+	+	-	-	+	-
2R1b	-	2.7µm	-	+	+	+	+	+	-/+	-/+	+	-	-	-	+	+	-	-	+	-
2R2b	-	4.1µm	-	+	+	+	+	+	-/+	-	+	-	+	+	+	+	-	+	+	-

TABLE 8: Morphological and biochemical characterization of freshwater isolates.

Strain	Carbohydrate											
	Glu	Fru	Man	Gal	Lac	Suc	Tre	Xyl	Sta	Manl	Gly	Sal
S1c	F	O	F	F	O	F	F	F	F	F	F	F
S4c	F	F	F	F	F	F	F	F	F	F	F	F
S61	O	O	NB	O	NB	O	NB	O	O	NB	O	O
R1a	O	NB	NB	O	NB	NB	NB	NB	O	O	NB	NB
R1b	O	O	O	O	O	O	O	O	NB	NB	NB	NB
R3b	O	F	O	O	O	O	O	O	NB	O	O	F
2S3b	O	O	O	O	NB	NB	NB	O	NB	NB	NB	NB
R3c	Fg	Fg	Fg	Fg	Fg	Fg	Fg	Fg	Fg	Fg	Fg	Fg

TABLE 9: Carbohydrate fermentation analysis of freshwater isolates. F = fermentation; O = oxidation; NB = no breakdown; g = gas production.

1.4.3 Antibiotic Sensitivity

The sensitivity of six freshwater isolates to a variety of antibiotics was determined using the multodisk technique at 30°C for 48h. Novobiocin, an antibiotic affecting cell wall biosynthesis and polymixin β-sulphate, active against the cell surface were assayed to find their respective minimum inhibitory concentrations (MIC) against strain S61. Similarly, the MIC for the nucleoside 9-β-D-arabinofuranosyladenine (Ara-A) against strain S61 was calculated. The results are summarized in Tables 10 and 11. A mutant (US61 (iii)) resistant to 0.625mgml^{-1} novobiocin was isolated by uranyl nitrate mutagenesis (Section 2.2.2). This will be used in a further study, comparing the attachment ability to the wild type. The MIC for strain NS61-20b, a non-mucoid mutant of strain S61, against novobiocin reveals a slight decrease in sensitivity to the antibiotic. The concentration of the Ara-A needed to inhibit growth of S61 was 4mgml^{-1}. This is 5000 x the concentration used by Takiguchi et al., (1978) to inhibit growth of Sphaerotilus sp. Hence, the effect of a lugml^{-1} solution in YE media upon adhesion would appear, not surprisingly, to be minimal (Section 3.5.3).

1.5 Successional Development

Marshall et al.,(1971b) reported a succession of bacterial types colonizing a surface over a 24h period. Small rods were initially sorbed, followed by more diverse, larger types. A similar response was found by Marszalek et al., (1979). Using different test surfaces, a complex 2-tier microfouling layer was observed to occur. The rate of fouling depended upon the substratum used. In general, the composition of the microbial film becomes increasingly complex with time and varies with the nature of the intial surface (Gerchakov et al., 1977). Different surfaces are fouled at different rates, some being more selective for certain organisms than others (Dempsey 1981b).

Four different surfaces (glass, copper, perspex and polystyrene) were immersed in the river and removed at regular intervals. Attached bacteria were isolated in pure culture, gram stained, and the cell size measured. The results are expressed below (Table 12).

Antibiotic	Strain S61	S4c	R1a	RIb	RB1	RB2
Tetracycline (10μg)	+	+	+	+	+	+
Chloramphenicol (10μg)	+	+	-	+	+	+
Erythromycin (10μg)	-	+	+	+	+	+
Kanomycin (5μg)	+	+	-	+	+	+
Penicillin G (1.5 Units)	-	-	-	-	-	-
Cephaloridine (5μg)	-	-	-	-	-	-
Ampicillin (2μg)	-	-	+	-	-	-
Methicillin (5μg)	+	-	-	-	-	-
Rifampicin (2μg)	+	+	-	+	+	+

TABLE 10: Antibiotic sensitivity of freshwater isolates.
+ = positive effect of antibiotic.

Antibacterial Agent	Strain S61	NS61-20b
Novobiocin	0.625-0.312mgml^{-1}	0.781-0.469mgml^{-1}
Polymixin β sulphate	0.156-0.078mgml^{-1}	nt
Ara-A	4mgml^{-1}	nt

TABLE 11: MIC values for strains S61 and NS61-20b.
nt = not tested.

Surface	Length of Immersion (h^{-1})				
	5	24	48	72	96
Glass	9	17	31	45	54
Copper	4	12	16	31	47
Perspex	11	22	29	47	61
Polystyrene	7	15	32	40	60

Table 12: Number of different bacterial types attached to specific surfaces after varying times of exposure.

A steady increase in the diversity of cell types attached is observed with the high energy, glass surface. A similar response is also shown by both the perspex (hydrophobic) and polystyrene (hydrophilic) surfaces. However, the copper surface was fouled at a slower rate, showing less diversity than the other surfaces. After 5h, there was one organism common to all four surfaces, two organisms peculiar to copper only, and two on the glass. However, after 24 and 48h, the glass, hydrophobic and hydrophilic surfaces showed a similarity in the type of fouling community. At least half of the bacterial population observed on the copper is unique to that surface. All the surfaces shown the same types of colonizing bacteria after 72 and 96h. No unusual forms were observed. Isolates previously found only on the copper after 24h were subsequently found on the glass and hydrophobic surface after 72h. These included a high proportion of mucoid strains.

The results would verify the statement by Dempsey (1981b) that all surfaces are colonized, though by different bacteria at different rates. Initially, mucoid organisms appear to colonize copper surfaces in relatively higher proportions compared to other cell types. The polysaccharide may offer some form of protection to the cell from the copper toxicity, reducing the leaching rate of the paint and chelating the heavy metalions (Dempsey, 1981a). Alternatively, copper-utilizing bacteria may have been selected for conflicting reports exist about whether hydrophilic or hydrophobic surfaces are colonized more quickly (Pringle & Fletcher, 1983). Dexter (1979) indicated that attachment to high energy, hydrophilic surfaces becomes maximum with increasing exposure, whereas, Fletcher and Loeb (1979) found higher numbers attached to low energy, hydrophobic surfaces. Baker (1984) reported colonization of both hydrophilic

and hydrophobic surfaces at the same rate. This led to the suggestion that different sections of naturally occurring bacterial populations are the initial colonizers of different types of surface. When presented with a variety of bacterial colonizers, the influence of the surface charge is reduced. Microtopography may prove to be more important than the actual chemistry of the surface.

1.6 Bacterial Distribution Within the River

Within any natural environment there exists a number of different micro-environments. In an attempt to categorize the distribution of micro-organisms found in a river location, three different environments were selected. These were: 1) Air/Water Interface; 2) Middle River and 3) Riverbed. At each site, 20ml samples were taken, plated on YE agar and nutrient agar, and grown at both 10°C and 30°C.

Sampling Site	Total Number of Different Organisms	% Gram Positive
Air/Water Interface	82	7.4
Middle River	61	12.1
Riverbed	58	15.3

Table 13: Bacterial types found in three river micro-environments.

The results expressed above (Table 13) serve only as a rough indication as to the distribution of micro-organisms within one location. By far the greatest range of organisms was found in the air/water interface. However, gram positive bacteria are more commonly found in the gravel and sediment of a river, reflecting their apparent absence from immersed surfaces. Within any one of these three environments exists a multitude of smaller environments. Thus, the variation in population at different levels of the river reflect the differences observed on colonized surfaces.

SECTION 2 Cell Surface Studies

2.1 Introduction

As discussed in the introduction, microbial adhesion in aqueous environments is important both ecologically and economically. Yet, despite the attention paid to the adhesion process, little is known about surface components involved. Recent trends in the literature have indicated that "the cell surface" mediates adhesion; alteration of the growth conditions alters the surface characteristics and hence the degree of attachment. Information regarding the characterization of cell surface structures from adherent bacteria is, on the whole, lacking. Fletcher (1980a) stated that "much more information is needed on the composition of cell surface polymers and their adsorption and solubility properties". The most likely components to be involved in adhesion are the extracellular polysaccharides (EPS), membrane proteins and fimbriae, though a lectin-lipopolysaccharide binding interaction may also be possible.

Exopolysaccharides have been shown by a variety of techniques to be involved in some aspect of bacterial adhesion. Sutherland (1980) performed compositional studies on polymers isolated from adherent strains. The results revealed that no unusual constituents were found in the polymers tested, though it was suggested that the apparent absence of O-acetyl groups might be important.

Pronase treatment, shown to remove (and prevent) attached cells (Fletcher & Marshall, 1982) indicated the possible involvement of surface proteins in adhesion. Pringle *et al.*, (1983) compared the outer membrane proteins of a non-mucoid strain of *Pseudomonas fluorescens* with a mucoid mutant. Little difference could be detected between the two membrane profiles.

The involvement of other components in bacterial adherence is rare in aquatic systems. Adhesion by, for example, holdfast material in species of *Caulobacter* (Poindexter, 1964) occurs only after 24h. Fimbriae are important for adhesion *in vivo*, yet there is little evidence to suggest their involvement in the natural environment. Weiss (1973), however, demonstrated that adherence of *Sulfolobus* sp to sulphur deposited in acid hot springs was by means of pili/fimbriae.

2.2 Characterization of EPS Isolated from Adherent Bacteria

2.2.1 Isolation, Purification and Hydrolysis of Polysaccharides

Cells were grown in YE media supplemented with 1% carbon source (glucose) in shake flask cultures. Occasionally, cultures were grown on 1/10 solid YE media. EPS was precipitated from the cell free supernate after 96h growth. After removal of salts, the product was lyophilized and subsequently analysed.

The results shown (Table 14) indicate that as was previously reported, (Sutherland, 1980), no unusual sugar monomers are found in the polysaccharides. Sugars found to occur most frequently are D-glucose, D-galactose and D-mannose. The majority of polymers have a uronic acid component. In some cases this appears to be D-glucuronic acid, though in others no further identification was made. The anionic material from four of the isolates (R3b, R3c, 2.96.1 and 4.5.1) did not co-chromatograph with either glucuronic or galacturonic acid. Included was the non-mucoid isolate, R3c, used for an attachment assay (Section 3.2).

An initial study by H.P.L.C. analysis has revealed that one of the polymers (RB2) may possibly have mannuronic acid as a component (A. Kennedy, personal communication, Appendix 1). The advantages of H.P.L.C. are that i) a rapid micro analysis can be performed: ii) optimized separation of residual components occurs: iii) no prior derivitization of the polymer is required (previous studies have all involved derivitization of the starting material, possibly causing inaccurate analyses): iv) trace components not detected by the paper chromatography technique may be recognized.

2.2.1.1. Non Carbohydrate Components of the Polysaccharide

Non carbohydrate moieties such as O-acetyl groups and ketal-linked pyruvate have been shown to be present in varying amounts in polysaccharide isolated from both freshwater and marine bacteria. Sutherland (1980) reported an apparent absence of O-acetyl groups from several preparations assayed, whereas relatively high amounts are observed in Table 15. Pringle *et al.*, (1983) described amounts ranging between 5-10% total carbohydrate. Similarly, pyruvate has been reported in varying amounts (Sutherland, 1980 and 1983). Values for total carbohydrate and protein content fluctuate greatly due to incomplete drying of the polymer (Table 16). The results expressed

Strain	Neutral Sugars						Uronic Acid
	D-Glucose	D-Galactose	D-Mannose	L-Fucose	L-Rhamnose	N-Acetyl Glucosamine	
S1c	+	+	+	-	-	-	+(glucuronic)
S4c	+	+	+	-	-	-	+
S61	+	+	+	-	+	-	+(glucuronic)
RIa	+	+	+	-	-	-	+
RIb	+	+	+	+	-	-	+
R3b	+	+	-	-	-	-	-
R3c	+	-	+	-	-	-	-
2S3b	+	+	+	+	-	-	-
RB1	+	+	+	+	-	-	+
RB2	+	+	+	-	+	-	-
2.5.1	+	-	+	-	+	+	+
2.5.5	+	+	+	+	-	-	-
5.24.2	+	-	-	-	+	-	+
3.48.2	+	+	+	-	+	-	+
2.72.3	+	+	-	-	-	-	+
2.96.1	+	+	+	+	-	-	+
GR(4)	+	+	+	+	-	-	-
4.5.1	+	+	-	+	-	-	-

TABLE 14: Monosaccharides identified in exopolysaccharides purified from attached freshwater bacteria.

Carbohydrate Fraction

Strain	% Pyruvate	% Acetate	% Uronic Acid*
S61	2.3	18.3	11.5
RIa	2.1	14.1	9.8
RB2	1.0	8.0	1.2

TABLE 15: Chemical composition of the carbohydrate fraction of EPS samples. Values of each derivative are expressed as a percentage of the total carbohydrate.

* A Kennedy, personal communication.

Polymer Composition

Strain	% Carbohydrate	% Protein
SIc	60.5	10.2
S4c	48.0	8.2
S61	72.0	12.0
RIa	80.0	16.4
RIb	64.0	4.4
R3b	92.0	3.6
2S3b	51.5	15.0
RB1	76.0	13.4
RB2	64.0	13.2

TABLE 16: Main components of adherent polysaccharides. Each value represents the mean of three separate assays.

should only be taken as an approximation for the actual value. Hence, Fletcher (1980a) reported a protein content of between 50 and 80% for a polymer isolated from an adherent marine pseudomonad strain.

2.2.2 Mutagensis

Production of EPS by strain S61 results in large mucoid colonies on solid media. The appearance when grown on YE media with 1% added glucose is shown in Plate 2a. Examination by microscopy and indirectly by centrifugation indicates the presence of a capsule. In order to evaluate the role of polysaccharides in adhesion, it is important to compare the attachment abilities of the mucoid wild type with a non-mucoid mutant.

Attempts were made to isolate mutants defective in EPS synthesis using standard mutagenic procedures. This proved to be unsuccessful despite repeated attempts using UV, gamma and uranyl nitrate irradiation. Mutants were not found to arise spontaneously, nor by prolonged growth in a chemostat.

However, non-mucoid mutants were produced eventually by NTG mutagenesis. One of these isolates, designated NS61-20b, was used for further study. Growth of the mutant under similar conditions to the wild type is shown in Plate 2b. The mutation proved to be stable, with no reversion to the wild type condition occurring despite repeated sub-culturing.

Because there is no tool for selection, mutants defective in EPS production must be isolated on the basis of morphological appearance. This can often be a severe handicap, as gross changes in the colonial appearance are needed before mutants can be categorically identified.

Further mutants were isolated, showing resistance to the antibiotic novobiocin. Novobiocin can be used to effect in the selection of mutants with altered cell surface components. Uranyl nitrate, an alpha particle emitter, was used to irradiate the cultures. A number of mutants were isolated fairly easily, none of them showing any morphological difference to the wild type. Growth on 1/10 YE agar plates supplemented with $0.625 mgml^{-1}$ novobiocin was used as the basis for selection, strain US61 (iii) being used for further study.

Plate 2: a) Growth of isolate S61 on 1/10 YE media supplemented with 1% glucose, 30°C, 48h.

b) Growth of non-mucoid mutant, NS61-20b under similar conditions.

2.2.3 Growth Curve Analysis

Growth of strain S61 in liquid media results in highly viscous cultures. Polysaccharide production and glucose utilization was compared for strains S61 and NS61-20b. In addition, strain S61 was grown in glucose-limited media. The production of polysaccharides under carbon limitation has been clearly demonstrated in Azotobacter vinelandii and Xanthomonas campestris (Sutherland, 1983a), so the possibility exists that other species may show a similar response. Glucose was growth limiting at a concentration of 0.065% (w/v), determined for continuous culture experiments (Section 5.3).

The respective growth curves are shown in Figs.13a, b and c. EPS was estimated by determining the total carbohydrate of cell free supernatants. Glucose utilization was followed by a glucose oxidase method (Boehringer), again in cell free cultures.

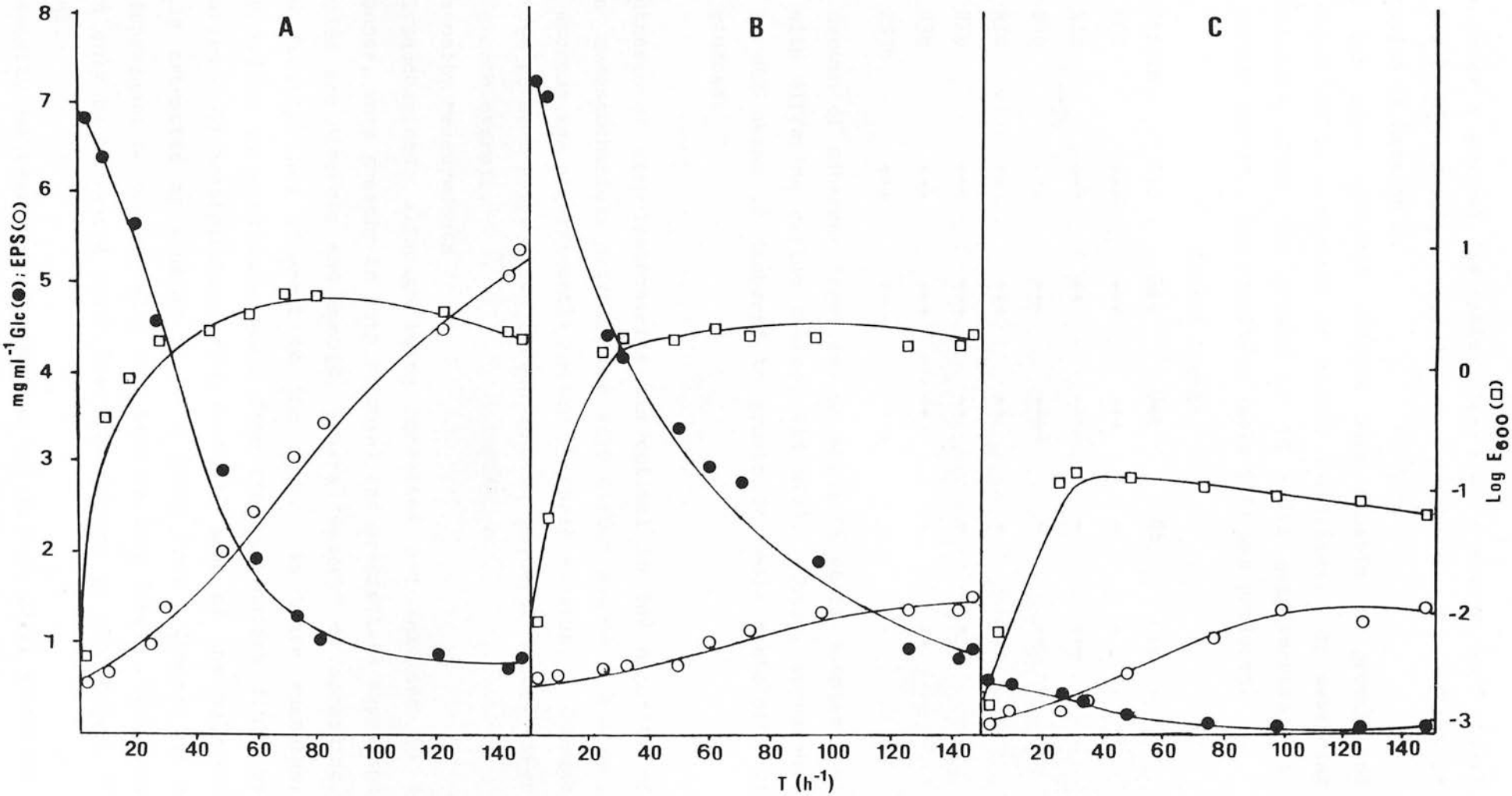
Polysaccharide production in strain S61 (Fig 13a) begins during the logarithmic growth phase and carries on into the stationary phase. Material is still being produced after 150h growth, though the rate of synthesis is decreasing. The maximum rate of glucose utilization also appears to the during the late log - early stationary phase.

Growth of the non-mucoid mutant, NS61-20b, is shown in Fig 13b. Much reduced levels of polysaccharide synthesis are observed. However, production follows a similar trend to the wild type, in as much as maximum production occurs during the late exponential - early stationary phase. Glucose utilization resembles that in the wild type.

The growth curve for strain S61 under glucose limitation is described in Fig 13c. Glucose utilization occurs until 50h; thereafter, negligible amounts were found in the media. EPS synthesis does occur, but at a low level, comparable to amounts produced by the non-mucoid mutant. Production appears to stop after 120h. A tenfold reduction in the total number of viable cells is also observed.

It would thus appear that EPS production for strain S61 is maximum at the late log - early stationary phase of growth at 30°C. The non-mucoid variant, selected on the basis of colonial appearance, does appear to have a reduced capacity for EPS biosynthesis. Moreover, the rate of glucose utilization and cell growth rate remain similar.

FIGURE 13: Polymer production (○); glucose utilization (●) and viable cell number (□) as a function of batch culture growth in YE medium, 120rpm, 30°C. (A) Strain S61 with 1% glucose supplement. (B) Strain NS61-20b with 1% glucose supplement. (C) Strain S61 with 0.065% glucose supplement.


A
B
C
mg ml^{-1} Glc (●) ; EPS (○)
Log E_{600} (□)
T (h^{-1})
8
7
6
5
4
3
2
1
-1
-2
-3
20
40
60
80
100
120
140

When grown under a glucose limitation, there is a slower cell growth rate with low levels of polysaccharide being produced. This effect is further studied in Section 5.

S61 and other adherent strains were capable of growth and polymer production on a variety of carbon substrates. By measuring colony dimensions after 48h growth on YE media supplemented with different carbon sources, the resulting Table (17) was produced.

	Carbon Source				
Strain	Glc	Gal	Suc	Ct	Gly
S61	+++	+++	+++	-	+
SIc	+++	++	++++	-	+++
S4c	+++	+++	++++	-	++
RIa	+++	+++	++	+	+++
RIb	+++	+++	++	-	+
R3b	+++	+++	++	-	++
2S3b	+++	++	++++	-	+

TABLE 17: Growth of adherent organisms on solid YE media supplemented with differing carbon sources (1% w/v). Colony appearance in all cases is compared to growth on media containing 1% glucose.

Synthesis of exopolysaccharide was optimal in the majority of cases when monosaccharides provided the sole carbon source. However, growth on sucrose was significantly better in three strains. Citrate did not prove to be a competitive carbon source; glycerol showed lower levels of growth overall.

2.2.4 Viscosity Measurements

Polysaccharides, although being harvested and analysed in a similar manner, vary greatly in both colonial and precipitate appearance. Some colonies are discrete and mucoid, others "watery" or spreading, and a few "crusty" and adherent to the agar. In these examples, difficulty arises in separating cells from the supernatant fluid by centrifugation. On precipitation with acetone, some of the polymers were easily extracted by winding on to a glass rod. Others had a granular appearance or occasionally were seen as long fibres. Polysaccharide could only be harvested under these conditions by evaporation of the solvent.

Viscosity measurements were carried out on 0.5% (w/v) solutions of cell free polymer. The response to varying shear rates is illustrated

in Fig 14. Most polysaccharide solutions tested were pseudoplastic; a decrease in viscosity occurred with an increase in shear rate. Pseudoplastic polymers have the property that as the random ground state is being disturbed, the molecules so arrange themselves as to prevent the least resistance to flow. Polymer chains thus exhibit alignments along the flow lines created by the shear fluid (Unsal, 1978).

Organisms in the natural environment are subject to varying shear rates. Yet, although the effects of shear can be studied in the laboratory, the results can only be approximations for those pertaining in situ. Complicating the assessment of the role of shear in the natural environment is the pseudoplastic nature of the polymer (Sutherland, 1983a). Viscosity can be altered by various factors, including changes in pH and in the concentration of ions present (Sutherland, 1980). The effect of temperature upon polymer viscosity depends very much upon the tertiary structure (Nisbet et al., 1984). Viscosity of disordered (random coil) polysaccharides decreases with increased temperature, whereas a conformationally rigid structure shows little temperature dependence.

2.2.5 Ionic Interactions

The interaction of ions with polysaccharides can result in either gelation or flocculation of the polymer. This may be of some importance with regard to bacterial adhesion. Polysaccharides as 1% (w/v) solutions were tested for gel formation by the addition of equal volumes of 2% (w/v) ion solutions (see Sutherland, 1980). Locust bean gum was also used to test for synergistic gel formation. The results are expressed below (Table 18).

Isolate	Al^{3+}	Mg^{2+}	Zn^{2+}	Na^{+}	Co^{2+}	Ca^{2+}	Mn^{2+}	Cu^{2+}	Fe^{2+}	Fe^{3+}	La^{3+}	LBG
SIc	P	P	P	P	P	P	P	P	P	P	P	-
S4c	-	-	-	-	-	-	-	-	P	-	P	-
S61	f	g	-	g	-	-	-	-	F	g	F	-
RIa	-	-	f	-	-	-	-	-	f	-	f	-
RIb	-	-	g	-	G	g	g	-	-	-	g	-
R3b	-	-	-	-	-	-	-	-	g	g	-	-
2S3b	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 18: Ionic interaction with polysaccharides from freshwater isolates. P = precipitation; G = gelling; g = slight gelling; F= flocculation; f = slight flocculation; LBG = co-gelling with locust bean gum after heating to 60°C and cooling.

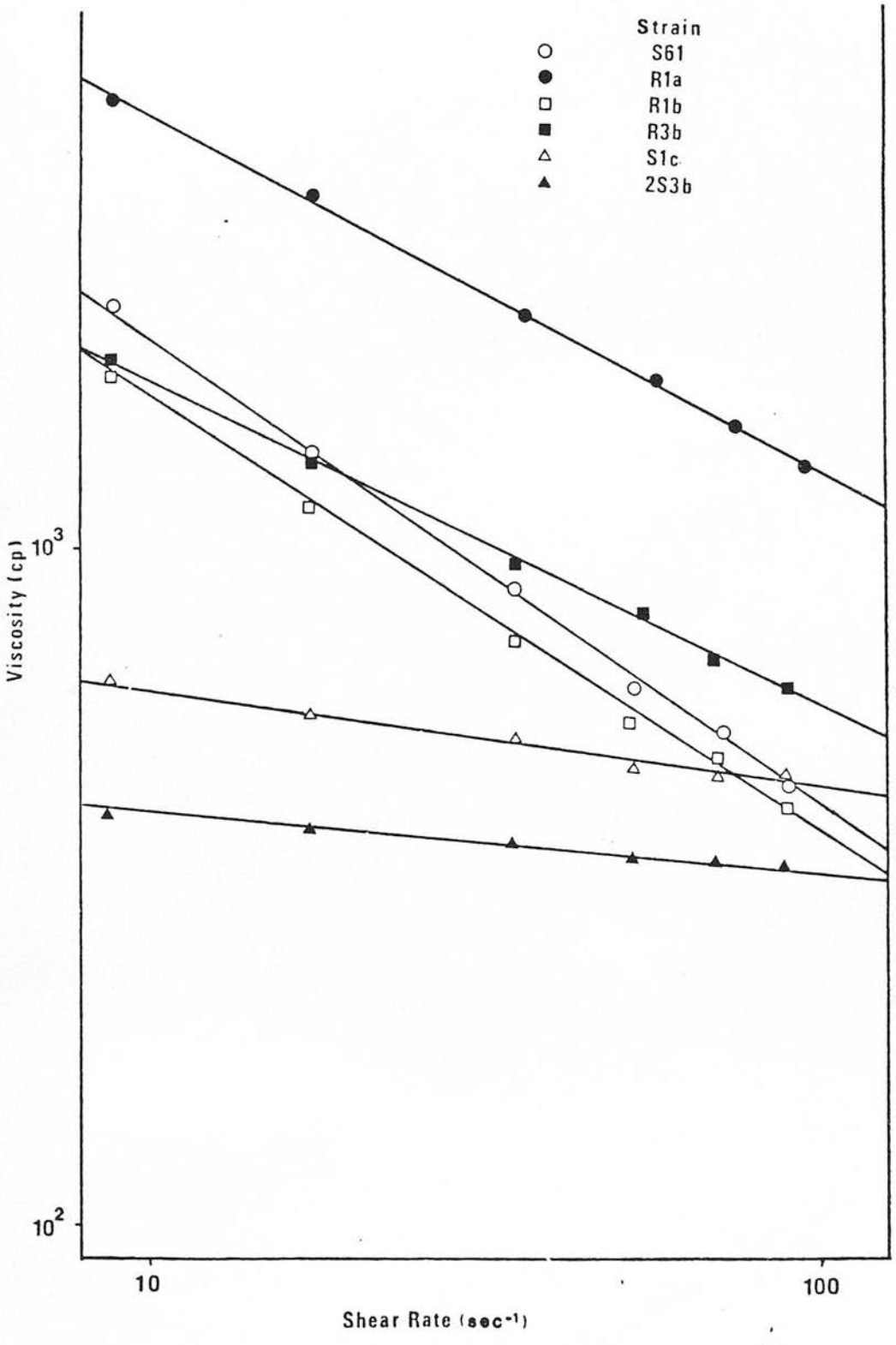


FIGURE 14: Viscosity of polysaccharides (0.5% w/v) from adherent freshwater bacteria.

Precipitation by hydrogen ions is unlikely. The pH of the water from which the bacteria were isolated was c.7.6-7.8. Strain SIc shows a precipitation pattern with all ions tested and RIb has a tendency to form slight gels with 5 ions. In some cases, the ions showed a strong affinity for the polymer and were difficult to remove from the precipitate. Grant et al., (1973) showed that the presence of Sr^{2+} or Ca^{2+} caused gel formation to occur with alginates. The strong binding was due to the presence of L- guluronic acid residues. This monosaccharide is rarely found in polymers and has as yet not been reported in any polymers isolated from adherent aquatic bacteria.

Co-gellation with locust bean gum did not occur with any of the polymers tested. To date, all of the experiments carried out on adherent polysaccharides have worked with a purified form. However, in the natural environment numerous organisms exist and interact with each other. Emphasis should therefore be placed upon the possible interactions of different polymers in solution.

Changes in the viscosity of polymers may be induced by the concentration of ions present. Sutherland (1980) showed that a drop in viscosity of polymer extracted from a marine isolate occurred with the addition of Ca^{2+}. The higher the Ca^{2+} concentration, the lower the viscosity. Viscosity measurements were performed with the polymer from strain S61, adding different concentrations of both Ca^{2+} and Mg^{2+} ions (Fig 15). The results indicate that both ions cause a decrease in polymer viscosity with an increase in ion concentration. This would appear to be true for polymers harvested from both marine and freshwater environments.

2.2.6 The Role of Divalent Cations for EPS Production

Marshall et al., (1971a) reported that Ca^{2+} and Mg^{2+} ions were important for maintaining bacterial adhesion. Further to this, Fletcher and Floodgate (1976) showed that adhesion could be prevented if cells were grown in media deficient in these two ions. The results expressed in Section 3.4 suggest that adhesion itself is not prevented, but microcolony formation. Low levels of polysaccharide were associated with adherent cells when grown under a Ca^{2+} and Mg^{2+} deficiency.

The yield of polymer, expressed as dry weight per mg dry weight of cells ml^{-1} is shown in Table 19.

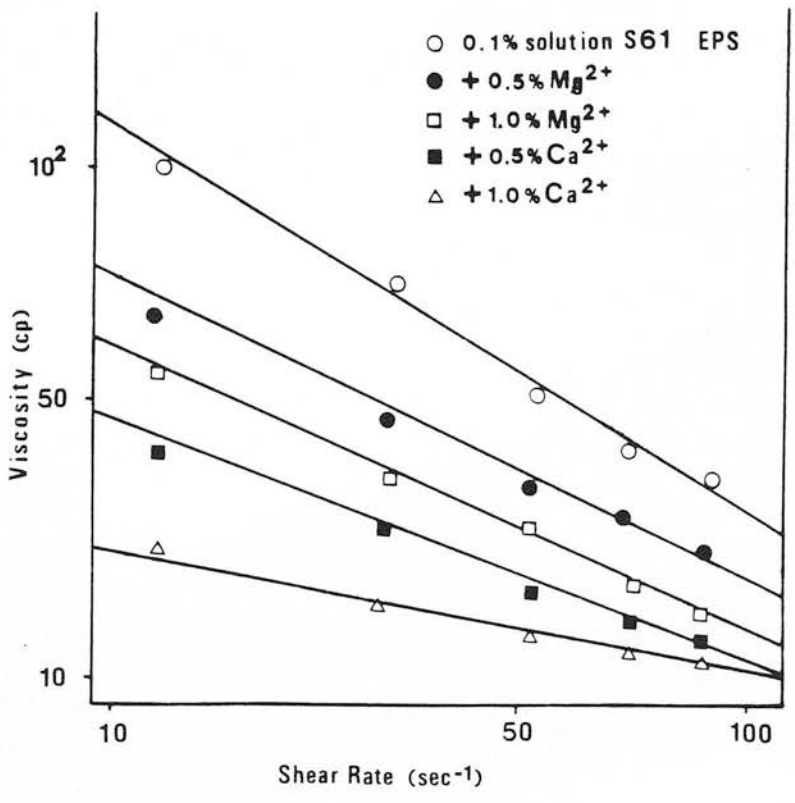


FIGURE 15: Effect of Ca^{2+} and Mg^{2+} ions on the viscosity of polysaccharide isolated from Strain S61.

Media	mg EPS $mg\,cell^{-1}\,ml^{-1}$
YE	0.266
YE minus Ca^{2+}	0.291
YE minus Mg^{2+}	0.860
YE minus $Ca^{2+} + Mg^{2+}$	1.147

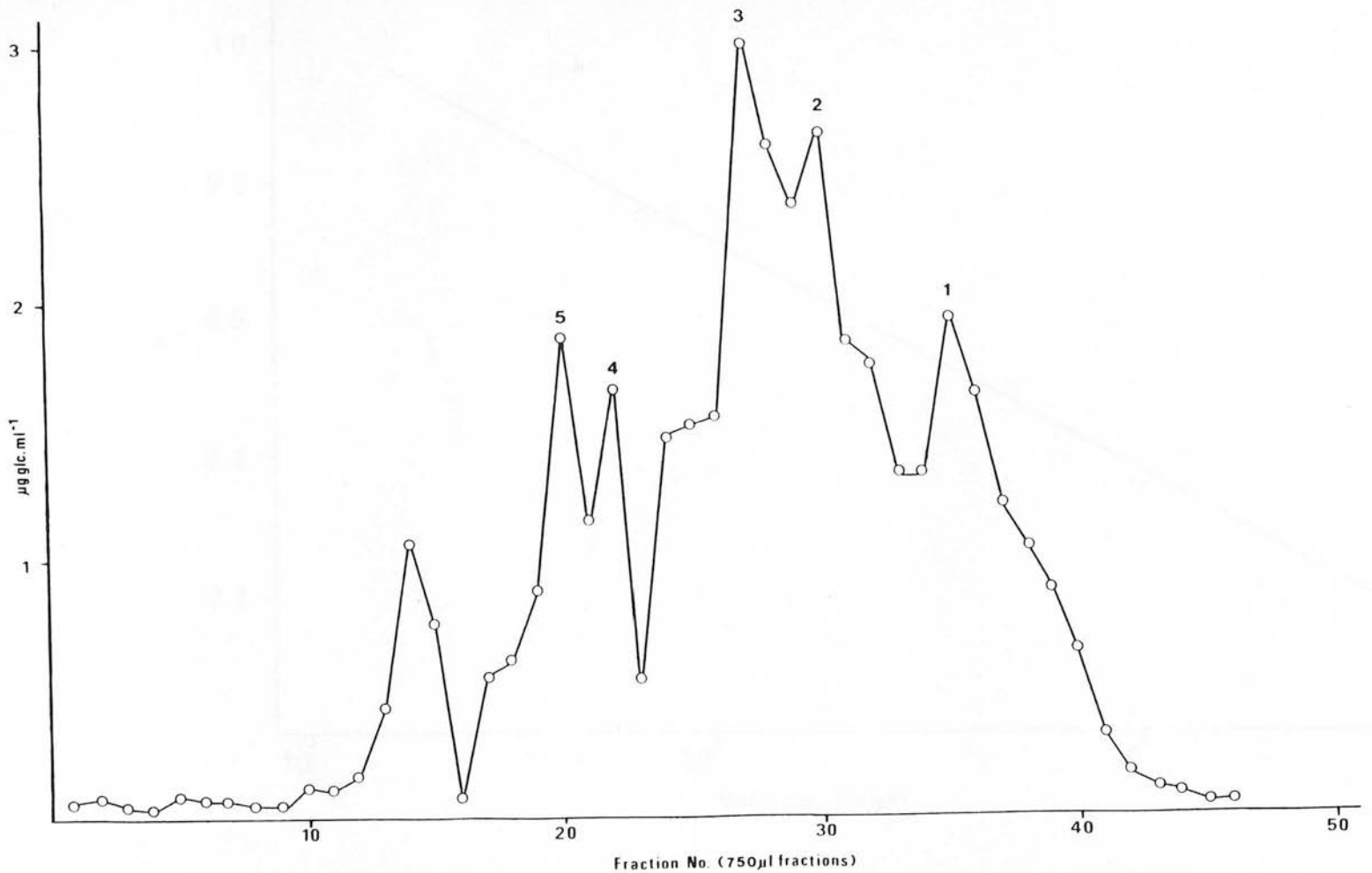
TABLE 19: The effect of Ca^{2+} and Mg^{2+} ions upon polysaccharide production in Strain S61 grown in YE media.

Similar amounts of polysaccharide are produced in both the YE media and the Ca^{2+} deficient media. However, there is almost three times as much produced in the Mg^{2+} deficient media and 4X the amount in Ca^{2+} + Mg^{2+} deficient media. This would suggest that the Mg^{2+} ions play an important part in the regulation of polysaccharide synthesis in Strain S61. The exact nature of this role remains unclear. Webb (1948) found that reduced levels of Mg^{2+} in the growth medium caused Clostridium welchii to increase the yield of EPS. However, Tempest et al., (1965) and Makovitz and Sylvan (1962) both showed that Mg^{2+} ions were essential for maximum synthesis of exopolysacchride. The reduced ability to synthesize polysaccharide was thought to be due to lower levels of enzyme precursors for EPS biosynthesis. Both ions would also appear to be important for maintenance of secondary and tertiary structure, explaining the lack of polymer accumulation (microcolony formation) on the substratum. Although described as Ca^{2+} and/or Mg^{2+} deficient, the media did contain trace amounts of the cations. Atomic absorption spectroscopy revealed that Ca^{2+} deficient YE media contained 4ppm Ca^{2+} and Mg^{2+} deficient media contained 0.29ppm Mg^{2+}.

2.2.7 Gel-Filtration

To determine the molecular homogeneity of EPS from Strain S61, it was applied to a sephacryl superfine 500 column. Five distinct peaks were eluted with distilled water (Fig 16). 500µl of a $2mgml^{-1}$ (w/v) solution was separated at a flow rate of $10cmh^{-1}$. A Kav plot (Fig 17) indicates that a family of polymers exist with a molecular weight spread between 1.4×10^5 and 3.2×10^6 (Table 20).

FIGURE 16: Chromatography of polysaccharide from Strain S61 on sephacryl S-500.


3
2
1
µg glc. ml⁻¹
10
20
30
40
50
Fraction No. (750µl fractions)
1
2
3
4
5

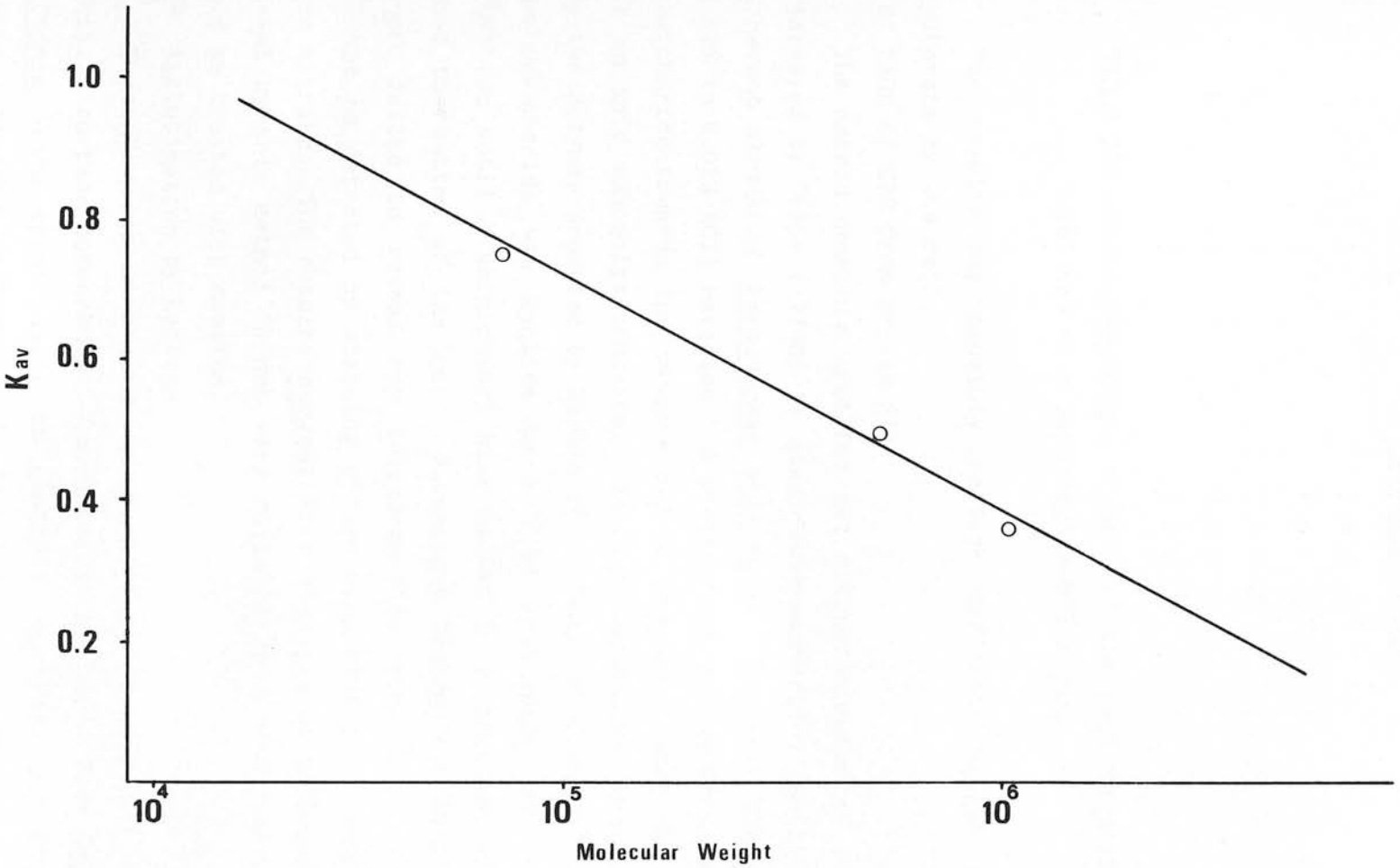


FIGURE 17: Kav values for dextran standards on a sephacryl S-500 chromatography column.

Peak	Molecular Weight
1	1.4×10^5
2	4.0×10^5
3	7.4×10^5
4	1.8×10^5
5	3.2×10^6

TABLE 20: Molecular weight values for the elution profile of S61 EPS on a sephacryl S-500 column.

The results may possibly reflect different stages of EPS biosynthesis by the cell.

2.2.8 PAGE of EPS from Strain S61

The method commonly used for gel electrophoresis of proteins was employed by Corpe (1970b) to study polysaccharides isolated from an adherent strain of Pseudomonas atlantica. Staining with alcian blue (1% in 0.01N HCl) revealed the presence of a blue-green band of polysaccharide towards the cathode end of the gel. This was judged to be an acid mucopolysaccharide. Similar techniques were used to study the polymer produced by Strain S61. 200µl of a $1 mgml^{-1}$ solution of polysaccharide was applied to a 7.5% acrylamide gel and the current run until a bromophenol blue marker in a separate well had reached the bottom of the gel. Subsequent fixing and staining of the gel failed to reveal any polysaccharide material. Proteins could not be detected by staining either with PAGE blue G-83 or by silver nitrate. The results suggest that analysis of polysaccharide material by this method is not very reliable, and that any results should be treated with caution.

2.2.9 Agglutination by Lectins

Three lectins were used in an attempt to identify exposed glycosidic surface components. Concanavalin A (Con-A) from Canavalia ensiformis binds specifically to glucosyl, mannosyl and fructosyl residues. Wheat germ lectin (WG) from Triticum vulgare is specific for N-acetyl glucosamine residues, and Soybean lectin (SB) from Glycine max shows specificity towards D-galactose and D-galactosamine.

2.2.9.1 Specificity Towards EPS Samples

4µl of a 0.1% solution of polymer was drawn into a capillary tube, followed by 4µl of a 0.01% lectin solution. Both ends of the

capillary were sealed and left for 24h at room temperature. A line of precipitation between the two solutions was an indication of a possible interaction.

2.2.9.2 Specificity Towards Whole Cell Preparations

All strains were grown in YE broth and tested for agglutination by spotting cells on a clean glass slide and adding equal volumes of each lectin. As a control, single drops of both cell culture and lectin solution were spotted. The results for both experiments are expressed below (Table 21).

Strain

	SIc		S4c		S61		RIa		RIb		R3b		2S3b	
LECTIN	EPS	WC	EPS	WC	EPS	WC	EPS	WC	EPS	WC	EPS	WC	EPS	WC
Con-A	-	-	±	±	±	±	-	-	±	±	-	-	-	-
WG	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SB	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 21: Lectin agglutinability against EPS and whole cell (WC) preparations.

A similar response was observed throughout for both EPS and whole cell agglutinability. Strains S4c, S61 and RIb all showed a slight response to Con-A. The remaining strains all failed to show any response irrespective of the lectin used or whether whole cells or EPS samples were used.

2.3 Outer Membrane Characterization of Strain S61

2.3.1 Isolation Procedures

Two methods were used in an attempt to isolate and study the outer membrane protein profile of Strain S61. The EDTA-Lysozyme-Sphaeroplast method of Osborn *et al.*, (1972) was found to produce less than half the amount of protein recovered from the PBS treatment, and was a longer procedure to perform. Both sets of samples showed similar characteristics when run on SDS-PAGE gels (gradient or non-gradient). Thus, for Strain S61, the PBS treatment followed by sarkosyl separation of inner and outer membrane was employed in preference to the Osborn technique. However, for other organisms, the Osborn method has been proved to be reliable.

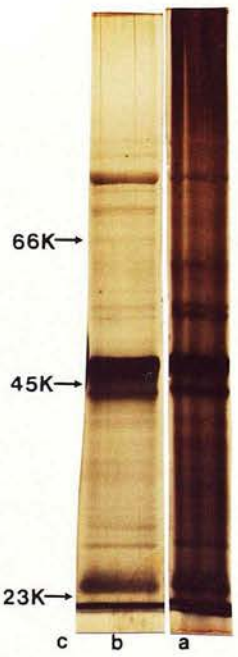


Plate 3: Gel electrophoresis of outer membrane proteins isolated from strain S61(a) and a non-mucoid mutant NS61-20b(b). Lane (c) represents protein markers (66, 45 and 23K daltons).

2.3.2 Outer Membrane Profile

The large number of polypeptides in stained SDS-PAGE gels of Strains S61 and NS61-20b are shown in Plate 3. Detecting changes in the protein profile may be hindered by the close proximity of bands leading to overlapping. Observation is either direct or by densitometric scanning. S61 possessed bands of molecular weight ranging from between c.90K to 20K daltons. A heavily staining band appears at the 45K region, and may possibly be a porin. Comparison of the wild type (S61) with the non-mucoid mutant (NS61-20b) reveals a similar protein profile. No additional proteins were observed in either strain. However, slight differences exist in the amounts of each protein present. The adhesive qualities of both strains may be compared favourably with regard to EPS production as no major change in the outer membrane appears to have been affected by the mutation.

The effect of varying nutrient limitations, temperature and dilution rate upon the outer membrane is discussed in Section 5.

2.4 Haemagglutination Assay for Fimbriae

Fimbriae are rarely involved in mediating adhesion between cell and solid surface in the natural environment. More commonly, they are found adhering to epithelial cells in the gut of living organisms (McCowan et al., 1978). Recently, Duguid and Old (1980) reviewed the adhesive properties of fimbriae. One aspect of these structures is of importance. Electrostatic repulsion could be reduced if the bacterial surface produced fine, hair-like projections. This would enable the cell to make contact with the surface whilst being held at the secondary minimum. The smaller the radii of curvature, the greater the degree of attraction.

Cells grown statically overnight in YE broth were washed and resuspended in saline. Equal volumes of the cell culture and a 3% (v/v) blood suspension were mixed in a spotting tray and left at 7°C overnight. Formation of a pellicle indicated no haemagglutination, whereas dispersion may indicate haemagglutination. This would need to be checked further by microscopy.

Of all the strains tested (SIc, S4c, S61, RIa, RIb, R3b and 2S3b), no haemagglutination was observed. The control organism, Saccharomyces cerevisiae showed strong haemagglutination. This would suggest that fimbriae do not play a role in adhesion of the

strains isolated. However, this means of assay may not be sensitive to microfibrils observed by SEM (Section 6).

SECTION 3 Light Microscopical Observations of Bacterial Adhesion

3.1 Introduction

A major problem in microbial adhesion studies has been the correlation of EPS production with attachment and growth of a cell to a surface. Reliable methods normally employed include SEM (Eighmy et al., 1983; Dempsey, 1981a & b), TEM (Jones et al., 1969; Fletcher & Floodgate, 1973) and epifluorescence microscopy (Weise & Rheinheimer, 1978; McCoy et al., 1981). Whilst showing polymeric material associated with adherent cells, they are both time consuming and subject to artifacts during sample preparation. Until recently, light microscopy has been used solely for enumeration and identification studies due to its low resolution. However, by application of a stain specific for carbohydrate material, the involvement of EPS in the adhesion process can be viewed by light microscopy.

Alcian blue has been used in many studies (Wiebe & Pomeroy, 1972) but has the disadvantage that the polysaccharide material must be sulphated. The limited resolution also means that the polysaccharides stained in this manner do not have their origin clearly demonstrated. By employing congo red as a stain specific for carbohydrate material, a method has been developed which allows EPS material associated with the cell and substratum to be observed by light microscopy. The method is a modification of a technique developed by Nogrady and Michaud (1979). Congo red, a diazo dye, is normally used to stain plant and fungal walls, the complex formed between dye and glucan being detected by changes in the absorption and fluorescence spectra (Wood, 1982). The dye interacts directly with intact β-D-glucans and can be used as an assay for strains possessing 1,4 β- and 1,3β-D-glucanopyranosyl units as well as the naturally occurring mixed linkage 1,4; 1,3β-D-glucan (Teather & Wood, 1982). The specificity of the stain however is not restricted to polysaccharides containing these two linkages. Using $1 mg ml^{-1}$ concentrations of congo red, Wood (1980b) observed weak interactions with other polysaccharide configurations. Elsinan, a polysaccharide containing 1,4- and 1,3-α-D- glucans also shows a weak interaction. This would suggest that there is a high degree of specificity

towards certain types of polysaccharide, related in some fashion to the configuration of the monomeric units and the linkages joining them (Wood, 1980b). By measuring changes in absorbance of a known amount of dye interacting with polysaccharide associated with attached cells, the results may help indicate the nature of the secondary and tertiary structure of the polymer concerned.

Quaternary ammonium salts are known to precipitate acidic polysaccharides (Scott, 1955), this fact being used in the initial mordanting of the specimen. The cetylpyridinium chloride also serves to nullify polyanions associated with the polysaccharide which would otherwise repel the congo red. Tween 80 is used to intensify the staining property of the congo red. Thus, when sampled in conjunction with the phenol sulphuric assay for total carbohydrate (Dubois et al., 1956), the association of polysaccharide material with adhering bacteria may be observed.

3.2 Attachment of Freshwater Isolates

Figure 18 is an illustration of the results obtained by this technique. Using a capsulate strain (S61) and a slime producing strain (Rla), a similar response was observed. Carbohydrate material associated with the glass surface accumulated gradually over the initial 5-6h exposure, then increased rapidly over the remaining 10h. In the control, there was s slight, non-specific adsorption of glucose to the glass surface. The accumulation of S61 cells and associated polysaccharide at 30°C, 120rpm, can be seen in Plate 4. A few isolated bacteria were observed after 4h exposure, with little associated carbohydrate material. Slight traces were observed at the 6-7h stage when microcolony formation started to occur. After 8h, the microbial aggregates are surrounded by a striking matrix of polysaccharide material. This increases in area after 11h and also in depth, causing loss of resolution after 13h. Microcolony formation appears to develop in a planar manner until a certain size is reached (cf 11h stage), whereupon development is subsequently away from the surface. These results are similar to the findings of Wardell et al., (1980).

The carbohydrate material associated with the attached RIa cells (Plate 5a) does not stain as intensely as that surrounding strain S61 (Plate 4). As previously discussed, this could give

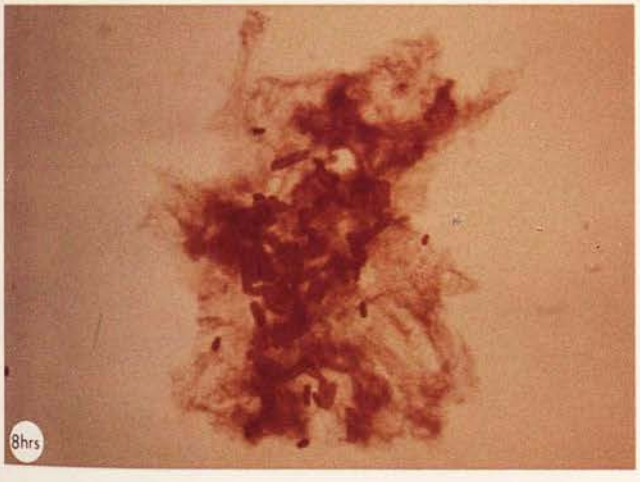
Plate 4: Attachment and microcolony formation of strain S61. Chemically clean glass slides were immersed in shake flasks (120rpm) of YE media at 30°C and removed at regular time intervals. Magnification X ca 1400.

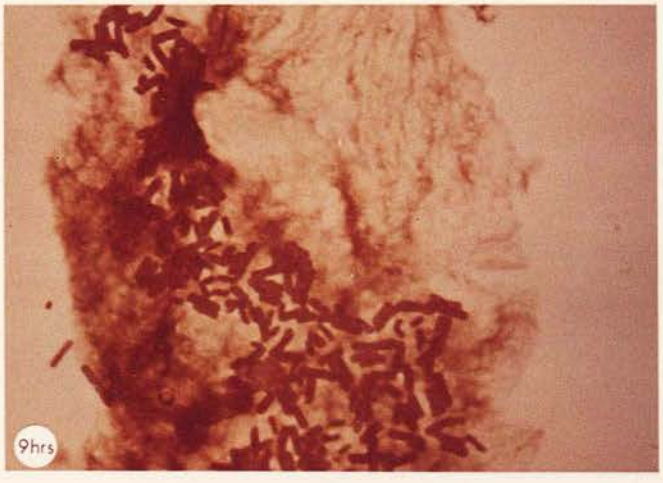

3hrs

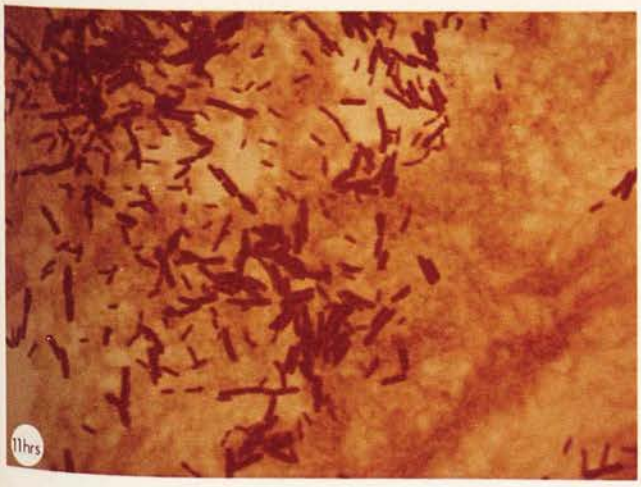

4hrs


6hrs


7hrs


8hrs


9hrs


11hrs

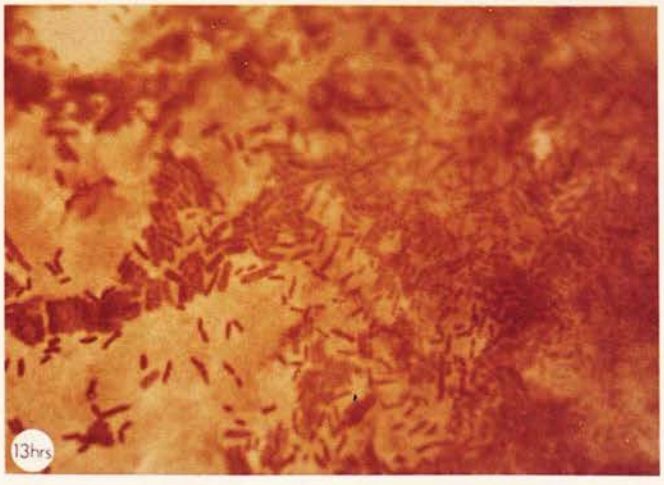
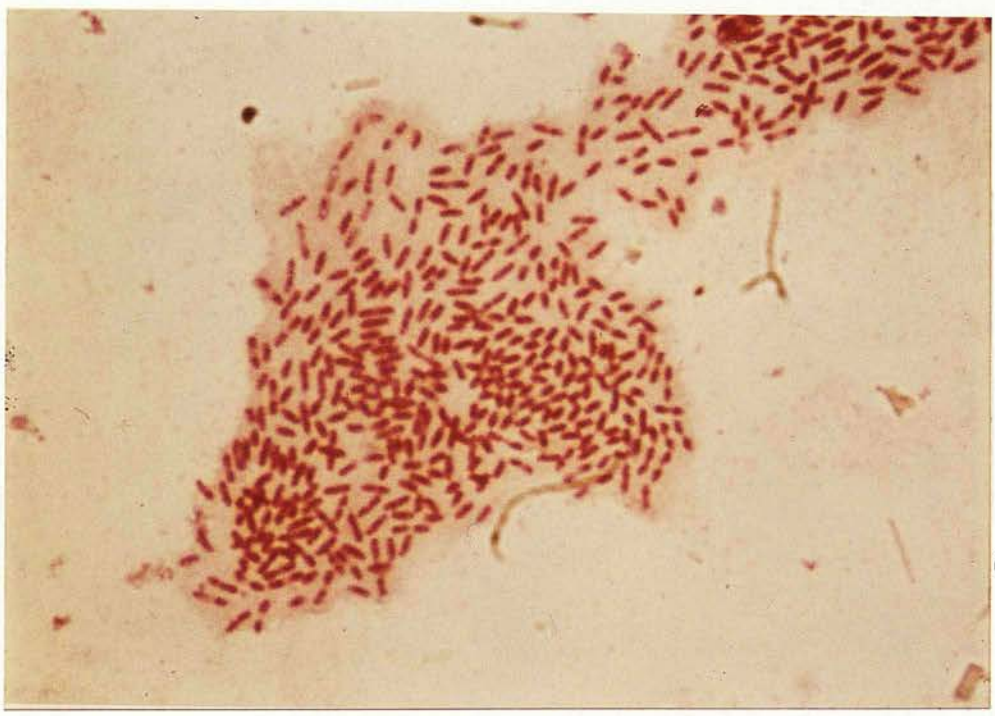
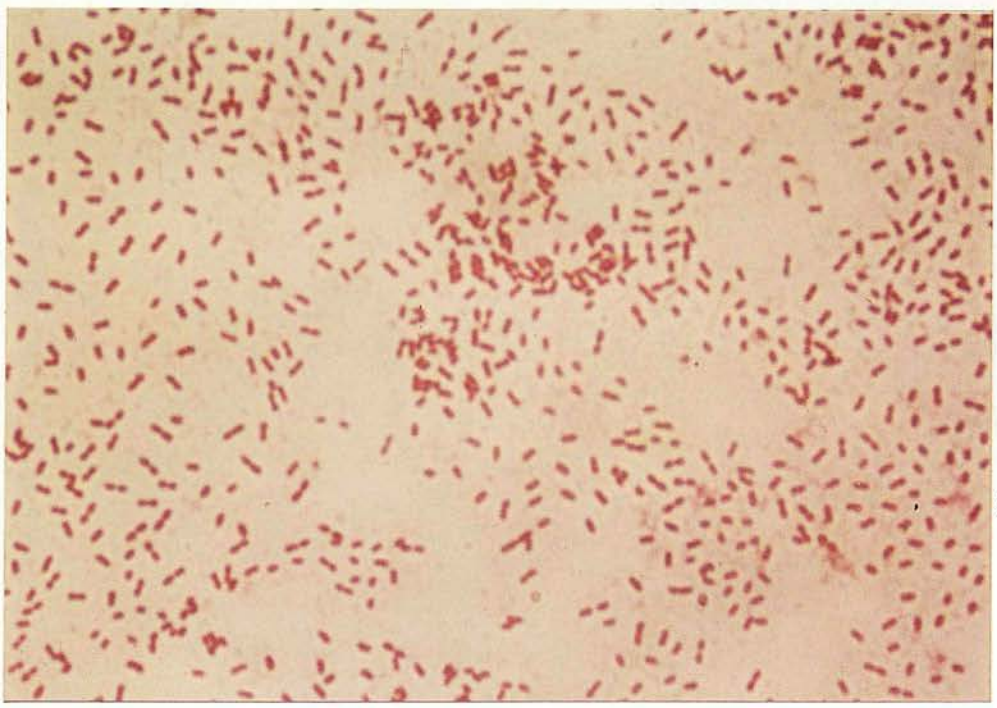

13hrs

PLATE 5: Microcolony development on a solid surface. Attachment of a mucoid organism, strain RIa, to chemically clean slides immersed for 10h (a) and 13h(b), and of a non-mucoid isolate, R3c after 13h growth (c). Magnification x ca 1100.

(a)



(b)



(c)

some indication as to the secondary organization of the polymers involved. Again, cells are observed to be enmeshed in a polymeric matrix. The accumulation of carbohydrate material on the substratum (Fig 18) assumes a sigmoidal shape associated with biofilm formation (Bryers & Characklis, 1982). After 10h, a compact microcolony is observed, being enclosed in a film of polymer. A further 3h growth and development reveals the microcolony to be larger, but still compact and enveloped by polymer.

A comparison of carbohydrate material accumulating on the glass surface was made between the two strains synthesizing EPS and a non-mucoid strain, R3c (Fig 18). Little in the way of polymeric material is formed on the glass surface, permitting cells to attach, but insufficient to allow microcolony formation to occur (Plate 5b). The bacteria were present on the surface individually in reduced numbers (10-fold reduction compared to S61 at similar stage)., or occasionally in pairs. There was no evidence for the presence of microcolonies.

3.3 Attachment of a Non-Mucoid Mutant

In order to determine whether or not this was a strain characteristic, the attachment of a non-mucoid mutant of strain S61 was observed under identical conditions. The strain, designated NS61-20b, was isolated by NTG mutagenesis, its growth characteristics being described in Section 2.2.3. Carbohydrate formation on the test surface (Fig 19) shows a similar trend to that of the non-mucoid isolate previously described (Fig 18). Very small amounts of polysaccharide are produced during the time course of the experiment, this being reflected in the microscopical observations (Plate 6). Cells are seen to attach, fairly evenly distributed on the surface, and increase in numbers with time. There is no siginificant difference in attached cell numbers at similar time intervals in comparison to the mucoid parent strain. However, microcolony formation is not observed, the cells being arranged individually or in pairs, occasionally as threes. This would suggest that the function of the polysaccharide material is not for the initial adhesion of cell to surface (Corpe, 1970b; Fletcher & Floodgate, 1973), but is involved in the development of a microbial film.

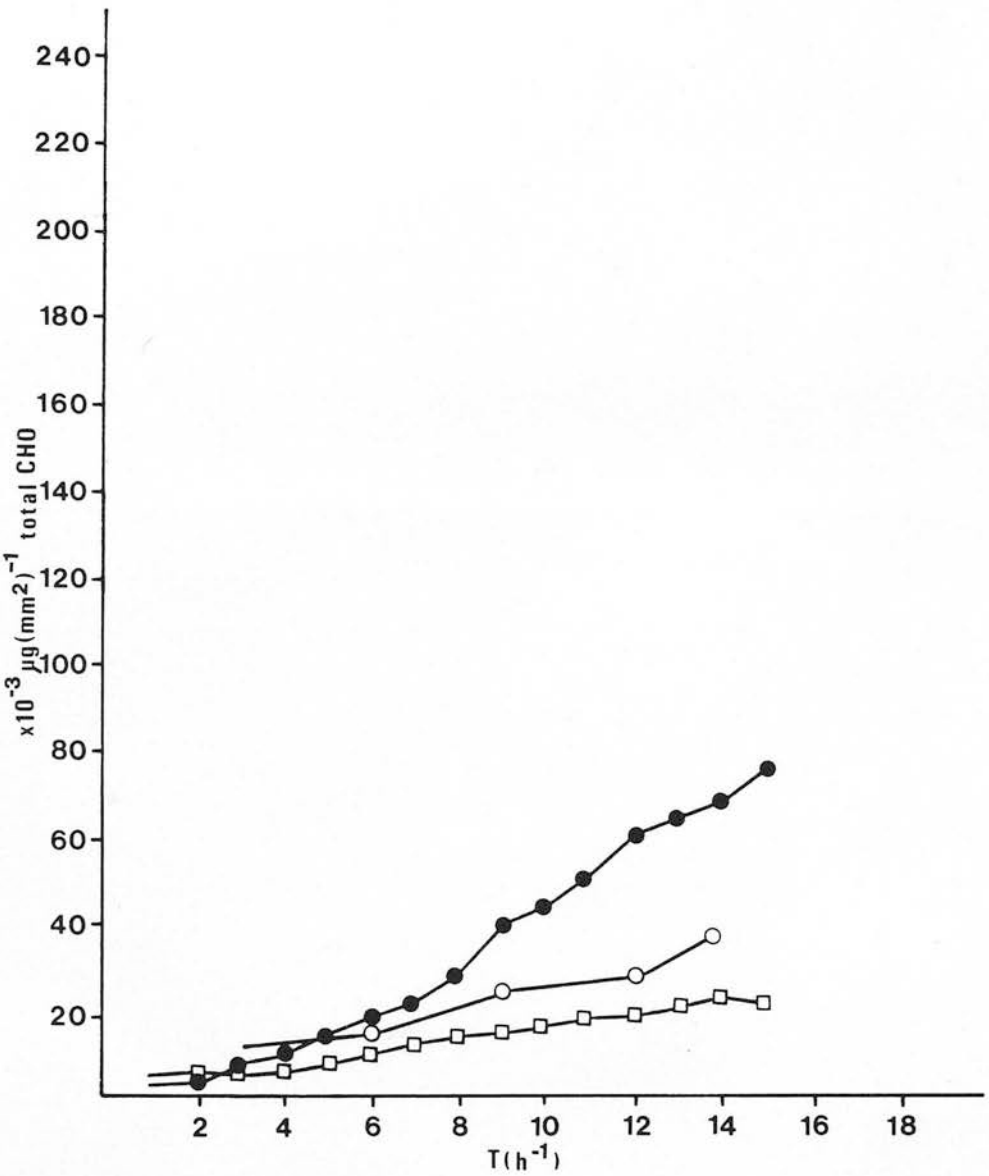
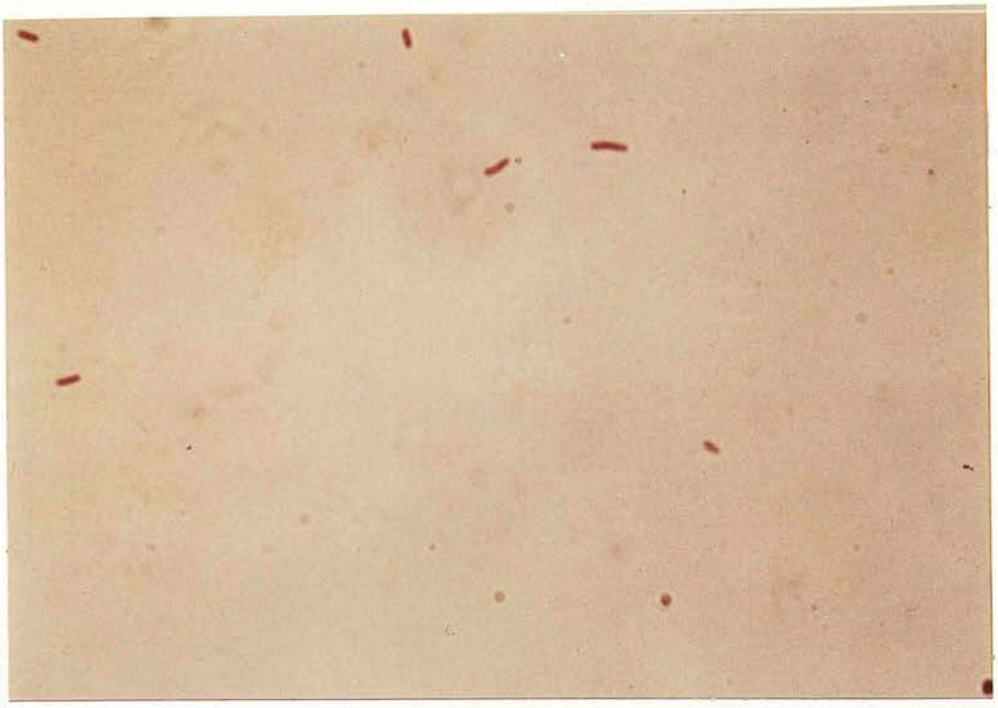
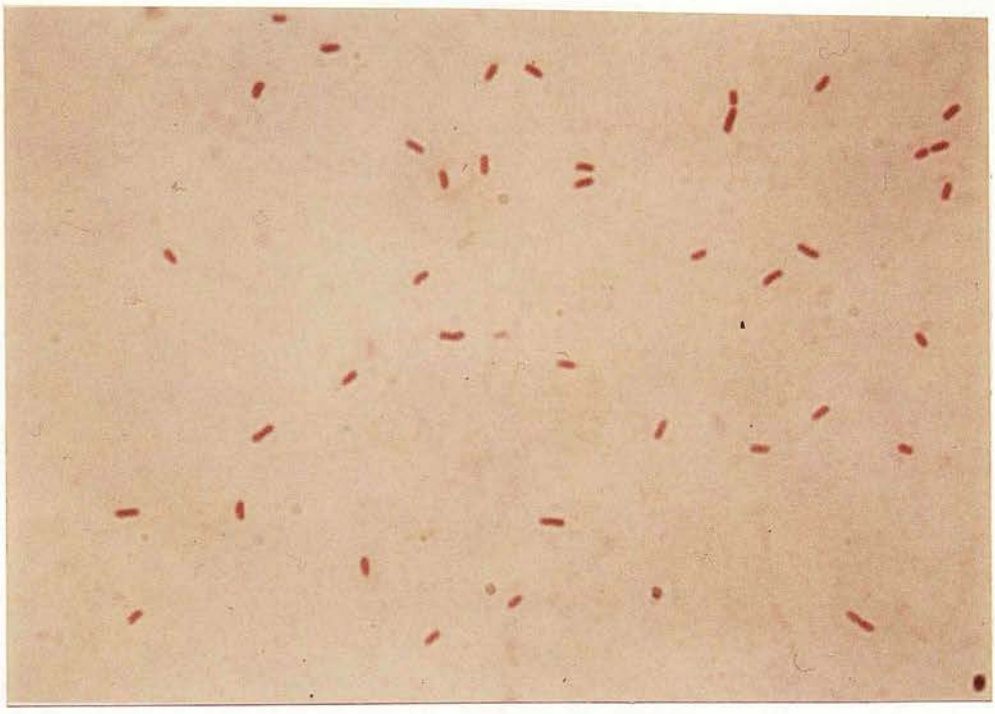


FIGURE 19: Polysaccharide adsorption to a glass surface. This was performed exactly as described in Fig 18, using a non-mucoid mutant NS61-20b (●) and strain S61 grown under glucose limitation (□). Again, a control using uninoculated medium was used for comparison (○).



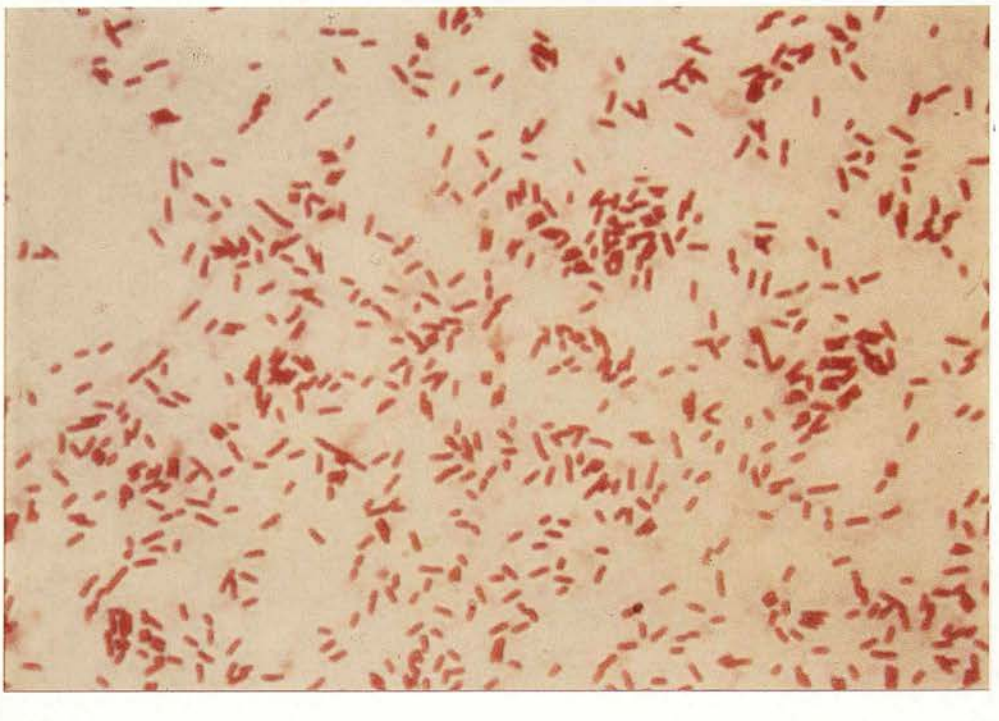
a



b

Plate 6: Attachment of strain NS61-20b, a non-mucoid mutant. Chemically clean slides were immersed for (a) 6h, (b) 8h, (c) 10h and (d) 13h in shake flasks at 30°C, as previously described. Magnification x ca 1100.

c



d

Plate 6: Cont'd

The accumulation of attached cells through the production of non-specific polymers has been described by Marshall et al., (1971a) as a three stage process. The first, or reversible stage is the initial attraction of the cell to the surface, usually through electrostatic forces. The second and third pahses are attachment, and growth and development. Polymer is produced in these two periods, enabling the cells to become firmly anchored, allowing them to grow and divide into microcolonies. Cells that cannot produce polymer may still adhere, but will not develop into colonies. They will therefore be at a selective disadvantge in the natural environment compared to polymer producing strains, as they will not have the benefits conferred by a polymeric matrix.

In both Fig 18 and Fig 19, there is little detectable difference in the carbohydrate levels found on coverslips immersed in cultures or sterile medium during the initial 4-5h exposure. Slight, non-specific adsorption of carbohydrate was found in the absence of bacteria, strengthening the concept of a requirement for a layer of organic material adsorbed to the surface prior to colonization (Geesey, 1982). Either the adsorbed organic layer is important because it will counteract the original physicochemical properties of the surface thereby favouring adhesion, or it will provide a layer to which a specific interaction between cell and surface can take place. In the former possibility, the surface free energy is reduced to a low negative value (Neihof & Loeb, 1972). If, however, a specific interaction is involved, the initial stages of adhesion could be time dependent. The results from Section 7 would suggest that for strain S61 there is a lectin-like response involved initially, the specificity being directed in part towards glucose and possibly mannose.

3.4 The Effect of Divalent Cations Upon Attachment

The importance of Ca^{2+} and Mg^{2+} ions to bacterial attachment has been clearly shown by Electron Microscopy. Fletcher and Floodgate (1976) demonstrated the involvement of primary and secondary polymers in adhesion, their dependency upon the presence of divalent cations. A disruption of the secondary polymer surrounding adhered cells was observed when transferred to Ca^{2+} amd Mg^{2+} deficient media. When grown under the deficiency, the polysaccharide

containing polymer associated with irreversible adsorption was not detected. These findings of Fletcher and Floodgate (1976) correlate well with the earlier studies of Marshall et al.,(1971a). However, neither set of results proves whether "secondary" polymer is actually produced, or is simply not being detected. By applying the congo red staining technique along with the carbohydrate assay, the involvement of Ca^{2+} and Mg^{2+} ions was investigated.

With cells grown in media deficient in both cations (Fig 20), there is a great reduction in the amount of carbohydrate material produced on the coverslip compared to media containing both ions (Fig 18). Cells were attached in reduced numbers, with no visible appearance of associated carbohydrate material until about 11-12h stage (Plate 7). Microcolony formation begins to occur, but is not extensive. Analysis of the media by atomic absorption spectroscopy (Section 2.2.6) revealed that there was trace amounts of Ca^{2+} and Mg^{2}present (4ppm and 0.29ppm, respectively). These small amounts could be sufficient to allow the cell to produce enough adhesive polymer to eventually form microcolonies. Omission of only Ca^{2+} ions from the media appeared to have no effect at all upon the production of adhesive carbohydrate (Fig 20), cells adhering in a similar manner and numbers to the normal media. However, media lacking Mg^{2+} ions showed a response intermediate to those of normal media (Fig 18) and Ca^{2+} and Mg^{2+} deficiency (Fig 20). The time taken for a significant increase in adsorbed carbohydrate amounts greater than the control, is almost twice as long. Cells in the normal YE media start to produce siginificant amounts of adsorbed material after 5h, whereas, with Mg^{2+} deficiency, this occurs after about 10h. From then on, the increase in adsorbed carbohydrate parallels the rate of formation of adsorbed carbohydrate in normal media, albeit the amounts are reduced. The results would suggest that Mg^{2+} are more important than Ca^{2+} in the accumulation of polysaccharide material on the surface. Possibly, they are involved in crosslinking the polymeric matrix, making it a more adhesive like material. Similarly, they could be involved in the crosslinking of polymer from one cell to the polymer of another, thereby helping to create an adhesive layer associated with cell and surface. Anwar et al., (1983)

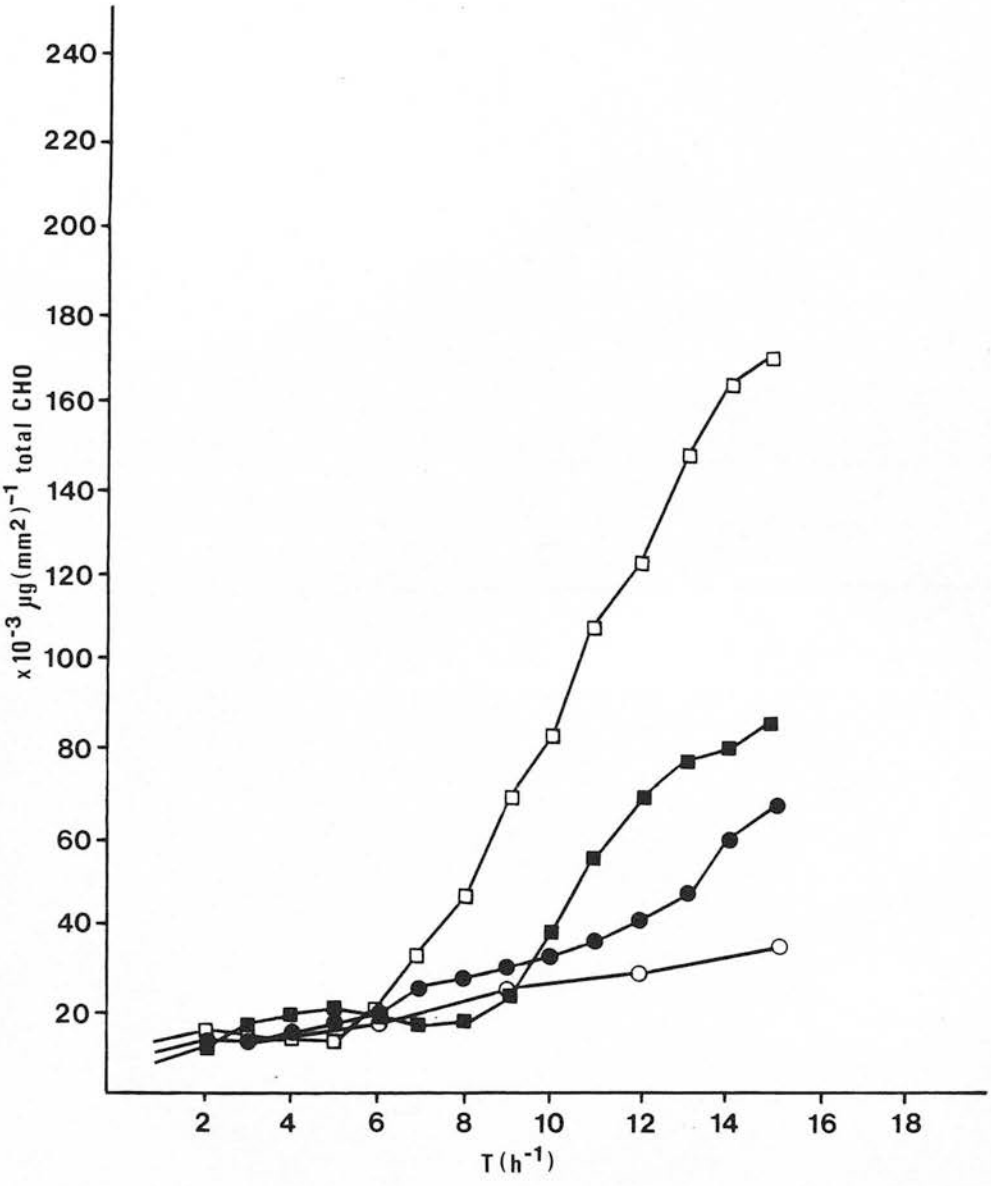
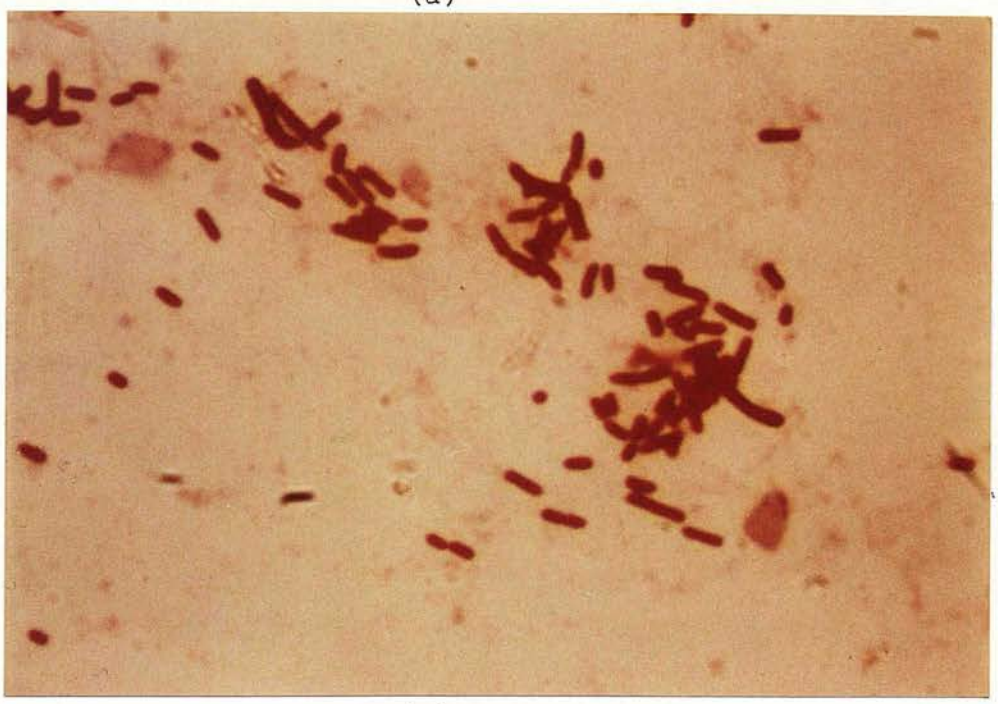


FIGURE 20: Polysaccharide adsorption to a glass surface. The effect of Ca^{2+} and Mg^{2+} deficiency on carbohydrate accumulation. (●) Ca^{2+} and Mg^{2+} deficiency; (□) Ca^{2+} deficiency; (■) Mg^{2+} deficiency and (○) control. Samples were assayed as previously described.

(a)



(b)

PLATE 7: Effect of Ca^{2+} and Mg^{2+} deficiency upon microcolony formation. Chemically clean slides were suspended in growing cultures of strain S61 for (a) 9h and (b) 12h. Magnification x ca 1100

demonstrated that in Mg^{2+} depleted cultures of Pseudomonas aeruginosa, an increased synthesis of the outer membrane protein H1 occurred. This conferred resistance to the antibiotics Polymixin B, Gentamicin and to EDTA. H1 was proposed to replace Mg^{2+} at the polyphosphate groups of the LPS molecules, stabilizing the outer membrane and protecting it from attack. Surprisingly, more polymer is produced in Ca^{2+} and Mg^{2+} deficient media than in the normal YE media (Section 2.2.6). With strain S61, it appears evident that Mg^{2+} and to a lesser extent, Ca^{2+}, is important for maintaining adhesion. Initial attraction to a surface is not affected by the lack of divalent cations, but their absence does prevent irreversible adsorption.

3.5 Effect of Changes in Growth and Substratum Conditions

3.5.1 Hydrophobic Surface

Hydrophobic surfaces have been shown either to prevent adhesion (Dexter, 1979) or to facilitate adhesion (Fletcher & Loeb, 1979). A glass slide covered in a silanising agent (Dimethyldichlorosilane solution) was assayed in the manner described for adherence of strain S61. The result indicated in Figure 21 reveals that carbohydrate production on the surface occurred at a greater rate, producing more material than on a higher energy glass surface. Associated with this increase in carbohydrate material, there was a corresponding increase in attached cell numbers; a $1\frac{1}{2}$ fold increase was measured on the treated surface compared to the untreated surface. The more hydrophobic a surface is, the lower the free energy (ΔG) of the system will be. This will favour bacterial adsorption as long as the surface tension of the suspending fluid is greater than that of the bacteria (Absolom et al., 1983).

3.5.2 Altered Glucose Concentration

Marshall et al., (1971a) reported the effects of increased glucose concentrations upon attachment. At high concentrations (30-70mgL^{-1}) the marine pseudomonad used was completely inhibited, whereas at 7mgL^{-1}, adherence was stimulated. The results shown in Figure 21, using strain S61 with a glucose concentration of 15gL^{-1} reveal that adhesion has barely been affected by the increase from the normal 10gL^{-1}. There is a steady increase in adsorbed material

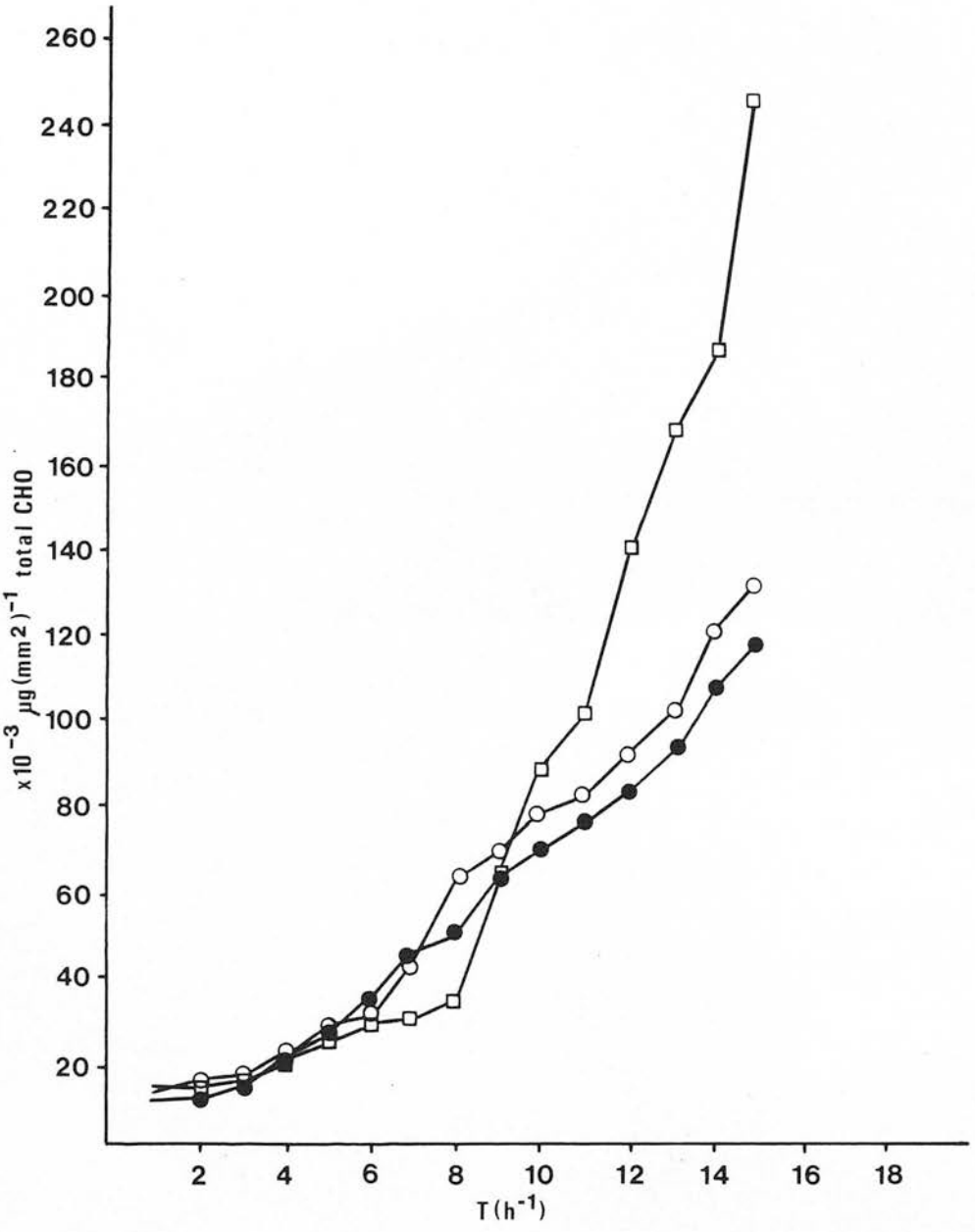
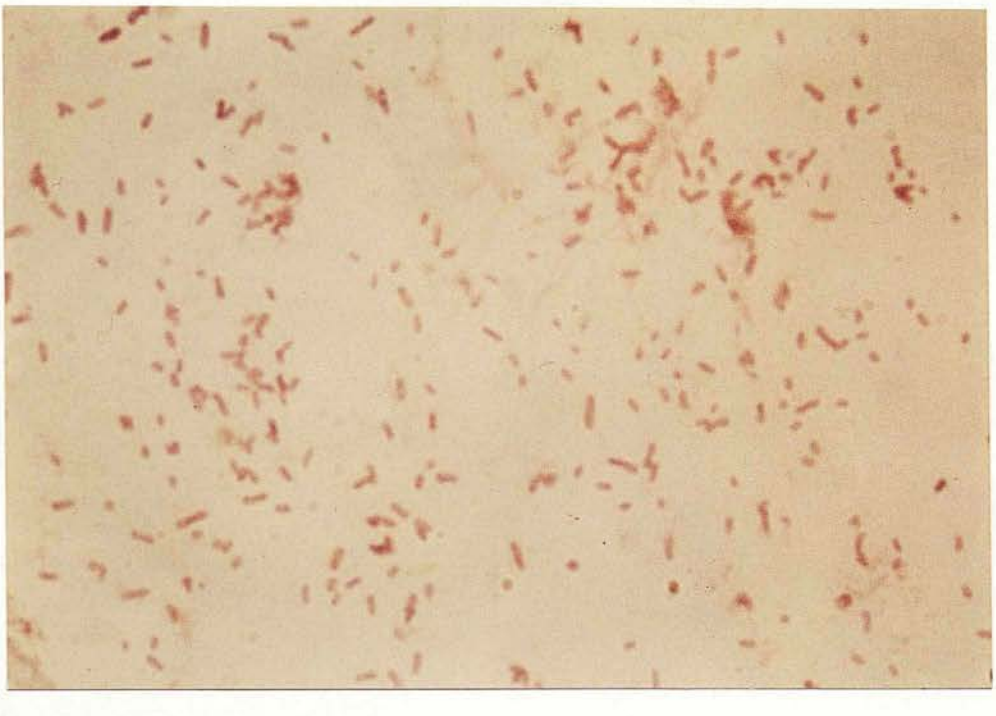


FIGURE 21: Polysaccharide adsorption to a glass surface. Attachment of strain S61 to chemically clean coverslips (□) pre-coated in a silanising agent; (○) immersed in YE media supplemented with 1.5% (w/v) glucose and (●) immersed in YE media containing $1 mgml^{-1}$ Ara-A.

for the whole time period, though the amount at 15h is less than for a similar time using 1% glucose.

Some organisms are known to produce polysaccharides under glucose limitations, for example, Azotobacter vinelandii and Xanthomonas campestris (Sutherland, 1983a). As was noted for X.campestris, the polymer produced under carbon limitation may differ chemically from the product of other nutrient limitations. The growth curves for strain S61 at a limiting glucose concentration of $650mgL^{-1}$ (Fig 13c) indicate that polymer is produced in the stationary growth phase. Assaying for carbohydrate material associated with attached cells, reveals that there is a 10 fold reduction in amount compared to cells grown with a 1% carbon source (Fig 19). If however these results are plotted on a larger scale, the shape of the graph is the same as that depicted in Figure 18, ie. the response is the same, but the values reduced. The actual number of cells attached is slightly greater than the number attached with a 1% carbon source. As can be seen in Plate 8, the cells at 10h have virtually no carbohydrate material associated with them. At 13h, cells are beginning to form small microcolonies, with a slight polymeric matrix surrounding them. This result adds weight to the idea proposed earlier that specific cell surface receptors are involved in the initial adherence of cell to surface. The role of the EPS would be to firmly 'cement' the cells to the surface and to allow microcolony formation to occur. The involvement of (specific) cell surface receptors has been previously suggested by Brown et al., (1977) to explain results obtained from continuous culture studies. Cells were found to adhere in greater numbers when grown under a glucose limitation than when grown a carbon excess; microfibrils were observed on the cell surface, but no surface polymer was apparent. Moreover, EPS production was abundant in the carbon-excess system, yet fewer cells adhered. The overall interpretation is that the cell surface receptors form a 'bridge' with, for example, the glucose molecules adsorbed by the inert substratum. In conditions of high EPS production the receptors are saturated by the polymeric material and not available for adhesion. Conversely, when grown in a carbon limited medium, the maximum number of receptor sites would

(a)



(b)

Plate 8: Attachment of strain S61 grown under glucose limitation. Chemically clean slides were immersed for (a) 10h and (b) 13h in YE media supplemented with 0.065% (w/v) glucose. Magnification x ca 1100.

be available to bind specifically. Scanning EM micrographs of strain S61 (Section 6.3) reveal the presence of surface microfibrils when grown in glucose limited medium. These were not evident however on the planktonic population, or the cells grown with an excess of glucose.

3.5.3 The effect of the Nucleoside 9-β-D-Arabinofuranosyladenine (Ara-A)

Yoshikawa and Takiguchi (1979) showed that a concentration of $0.78\mu gml^{-1}$ Ara-A, was sufficient to inhibit the growth of Sphaerotilus natans and other related adherent species. Using a final concentration of $1\mu gml^{-1}$ in YE media, the effect of Ara-A upon the attachment ability of strain S61 was studied. The results (Fig 21) indicate that attachment is not prevented. Correspondingly, there is no reduction in the amount of adhesive polysaccharide formed. The effect of Ara-A upon EPS production in S.natans was not tested, therefore it remains unclear as to whether a specific effect on polysaccharide synthesis was responsible for the inhibition of attachment. Ara-A appears to be selective, inhibiting the growth of S.natans and Beggiatoa species, yet failing to affect the growth of other bacterial species at concentrations exceeding $1mgml^{-1}$.

A point worth considering is whether or not the polymeric material observed to be associated with adherent bacteria is of the same composition as the material purified from the culture supernate. The literature reports an increasing number of examples where bacteria are being shown to produce more than one type of polysaccharide. Examples well documented include Rhizobium spp (Dazzo & Hubbell, 1975; Zevenhuizen et al., 1979) producing a low molecular weight β1→2 linked glucan; Agrobacterium rhizogenes (Gorin et al., 1961) and Alcaligenes faecalis (Hisamatsu et al., 1978). The possibility exists therefore, that many more species, capable of doing this, have so far remained undiscovered. There is no reason to suggest either, that these do not include adherent bacteria.

3.6 in situ Studies

A limiting factor in studies of microbial isolates from natural environments is the extrapolation of observations made in the laboratory to actual in situ events. To determine whether the

results obtained by this staining technique reflected the sequence of events in nature, chemically clean slides and coverslips were suspended in the river from which the bacterial strains were originally isolated. Sampling was conducted at fairly regular intervals up to 125h. The results obtained (Fig 22) indicate that for the first 70h, carbohydrate accumulated gradually on the glass surface. Thereafter, the amount of adsorbed polymer increased at a greater rate. This correlates well with the laboratory findings which also showed a slow initial build up followed by a more rapid accumulation later on in growth. As the mean temperature of the river was about 9°C, events would be expected to be slower than in the laboratory. Other factors contributing to a slower build up include a low nutrient concentration, greater flow rate generated on the glass surface, and a mixed culture of organisms competing for the surface.

Small rods proved to be the dominant form that adhered, though some larger rods and cocci were also observed along with associated polysaccharide (Plate 9). A few individual cells lare observed after 32h, along with an example of rosette formation. This could be similar to the example described by Moore and Marshall (1981) and Marshall and Cruickshank (1973). Cells of varying dimensions were observed to orientate themselves in a rosette pattern, being held together by amorphous holdfast material believed to be of a polysaccharide nature. The orientation of the cells was thought to be the result of one portion of the cell surface being more hydrophobic than the remainder of the surface. After 72h, more cell types can be seen, being surrounded by polymeric material. Accordingly, both the number and types of different bacteria attached to the surface has increased by 125h, with an accompanying increase in adsorbed carbohydrate material.

Slight modifications are needed for the rinsing step of the staining procedure when it is applied to natural habitats. The granular background makes the identification of organisms and associated material difficult and should be removed whenever possible.

The improved staining technique and associated carbohydrate assays can be useful methods for the study of bacterial attachment

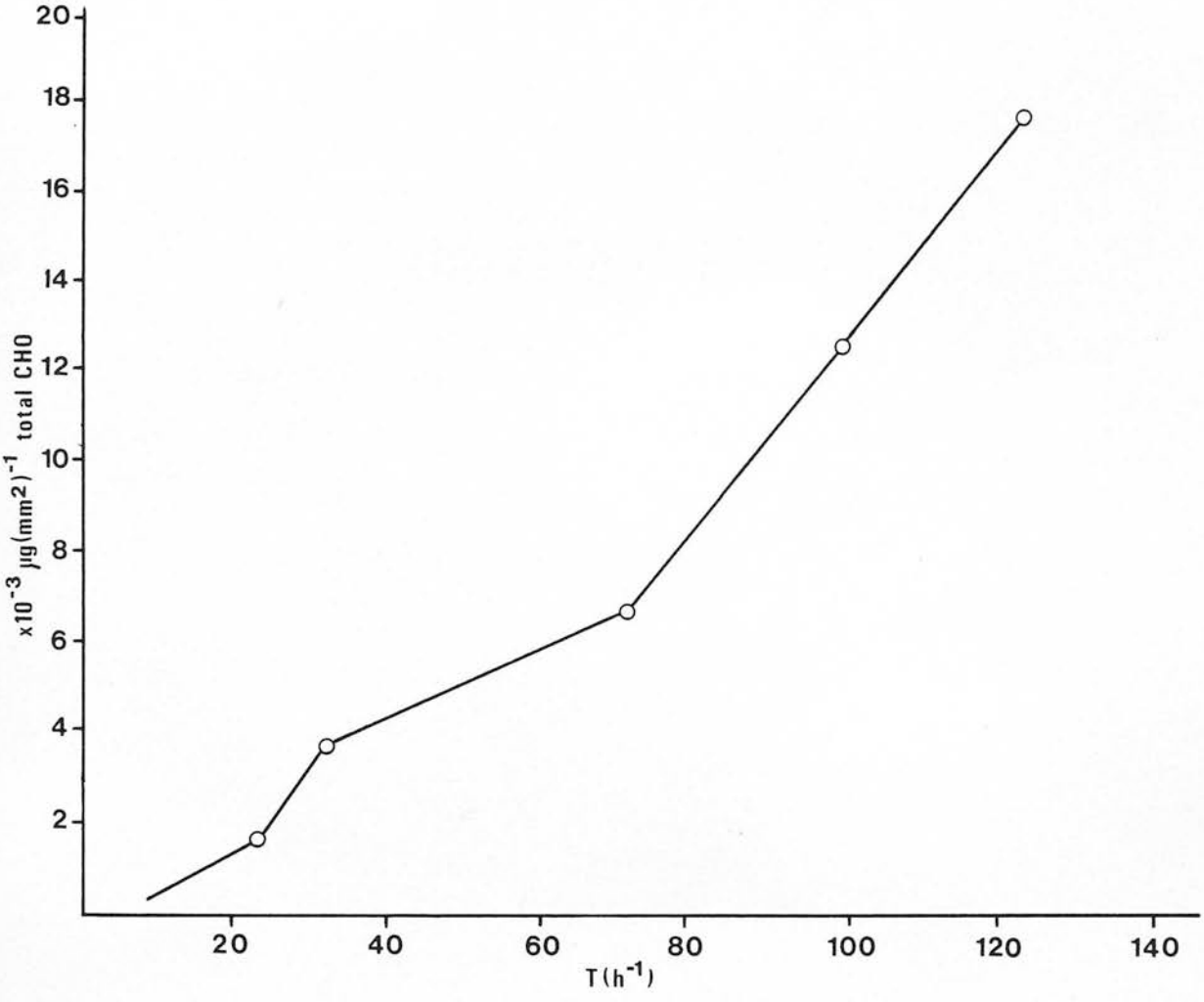
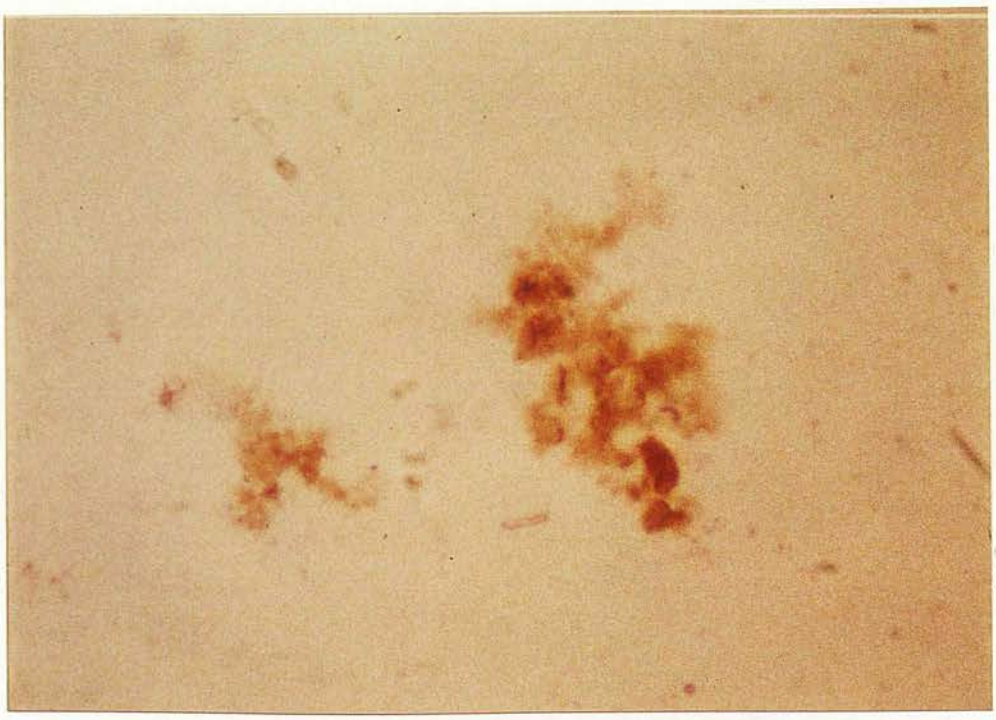


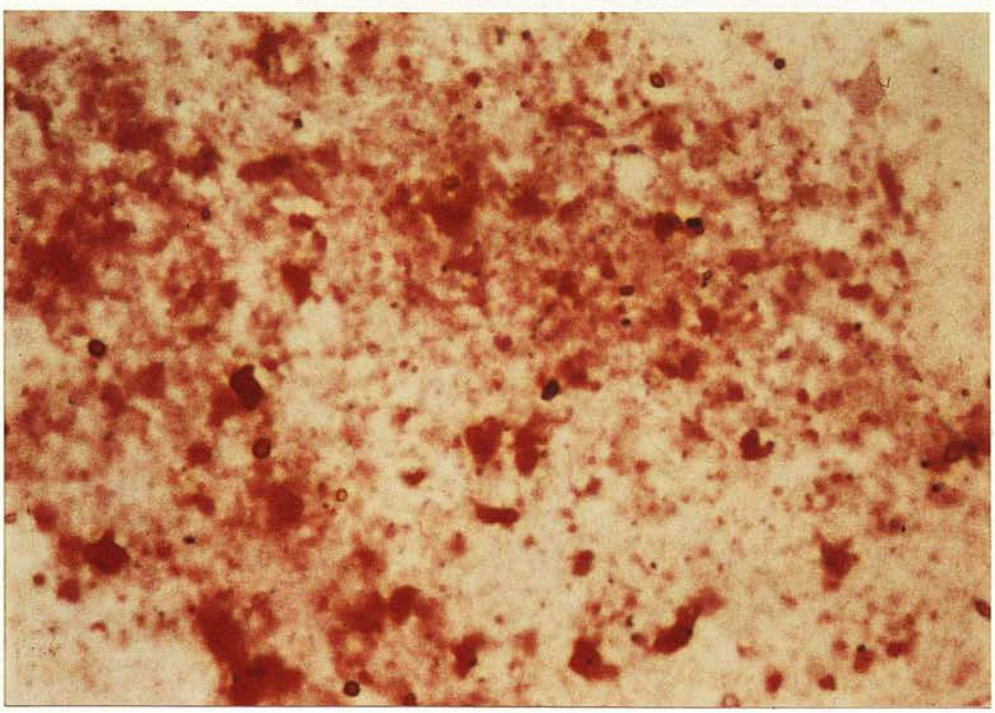
FIGURE 22: Carbohydrate accumulation on glass coverslips suspended in fast flowing river water, 9°C.

Plate 9: Attachment of micro-organisms to chemically clean glass slides suspended in a fast flowing river at 9°C for (a) 32h, (b) 72h and (c) 125h. Magnification x ca 1100.

(a)



(b)



(c)

and biofilm formation. Advantages include the fact that it is quick, easy to perform and can be used to study a variety of situations where the involvement of EPS can be investigated. Observations made under the light microscope can be correlated directly with the chemical assays and the techniques applied either in vitro or in situ. The results from the laboratory appear to reflect the events occurring in the natural environment and are expressed in a qualitative manner. Hence, the involvement of bacterial exopolysaccharide in the attachment process can be readily demonstrated.

SECTION 4 Parameters Affecting Adhesion

4.1 Introduction

The effects of various parameters upon bacterial adhesion can be estimated by counting the number of attached cells. Normal methods of enumeration include phase contrast microscopy, epifluorescence microscopy and SEM. The results however, only yield information about the distribution and morphology of the attached cells. Other methods that have been used include light section microscopy and ultrasonic interferometry, both of which estimate the thickness and acoustic attenuation of the biofilm. Radioisotope labelling which is a very sensitive method, has been employed on a few occasions, as have biomass measurements (eg. ATP generation, carbohydrate and LPS production, protein and nitrogen turnover). All of these methods are however, slow and/or expensive to perform. Plate counting, whereby cells are removed from a surface and serially diluted, is a quick and reliable method of tabulating the number of attached cells. Dempsey (1981a) used this procedure to evaluate the toxicity of antifouling paints against marine bacteria. His results indicated two general patterns of colonization subject to the type of paint used. The limitations of this method are that only viable cells are counted. Thus, cell counts will always be lower than the actual attached number. Paul and Loeb (1983) developed a rapid, inexpensive method for estimating cell numbers on a surface involving a fluorometric determination of DNA. This method was compared to conventional plate counts when studying the adherence of a marine pseudomonad to polystyrene surfaces. A close examination of the resulting graphs reveals that there is little difference in accuracy between the two methods; the fluorometric technique showing less variability between replicates than the plate counts. The plate count method is therefore a quick, easy, reliable technique for estimating levels of attachment *in vivo* where accurate cell counts are required.

4.2 Physicochemical Responses

Fletcher (1977) studied the attachment of a marine pseudomonad to polystyrene in an attempt to see whether attachment required a physiological response or whether the bacteria adhered to the

substratum spontaneously. Cell culture concentration, age, time, and temperature were all shown to be important factors governing adhesion, the results being described by a model based on physico-chemical adsorption. The freshwater isolate, S61, was used in a similar series of experiments to compare with the marine isolate. All points plotted are the mean values of triplicate readings plus or minus the standard deviation (σ n-1).

4.2.1 Effect of Culture Concentration

The results depicting the effects of culture concentration are shown in Figure 23a, b and c. Log phase cells were harvested from an overnight incubation, washed, and resuspended to the final volumes indicated. Chemically clean glass slides were immersed for 1h, removed, and the surface thoroughly swabbed. At a high culture concentration (Fig 23a), there is a gradual levelling off of attached cells. This is probably due to the glass surface becoming saturated. A ten fold decrease in culture concentration (Fig 23b) reveals that there is a steep increase in the number of attached cells with respect to the culture concentration. When the culture concentration is diluted a further ten fold (Fig 23c), there is a slow, but increasing level of attachment. These results suggest that as the concentration of cells in the suspending medium increase, the number of attached cells also increases. A point is reached however, when cells stop adhering to the surface. This is when the substratum has become saturated, preventing any further increase in attached cell numbers. Adhesion though will still be occurring, due to the continuous removal of cells with time and the occasional "sloughing off" of material, creating spaces for new cells to adhere.

4.2.2 Effect of Time Upon Attachment

The dependence of attachment upon time is closely related to the culture concentration. Using a concentration of cells that adhere in relatively high numbers after 1h (6.3×10^7 cells ml^{-1}), the effect of time allowed for attachment was studied. The effect of a ten fold reduction in culture concentration was also followed. At a high concentration of cells ($\sim 10^7$ cells ml^{-1}) there is a rapid increase in attachment with time (Fig 24a) whereas at the lower concentration, there is a more gradual rate of increase (Fig 24b). Thus, it would seem that attachment is influenced by both culture concentration and time. An increase in both factors should lead to

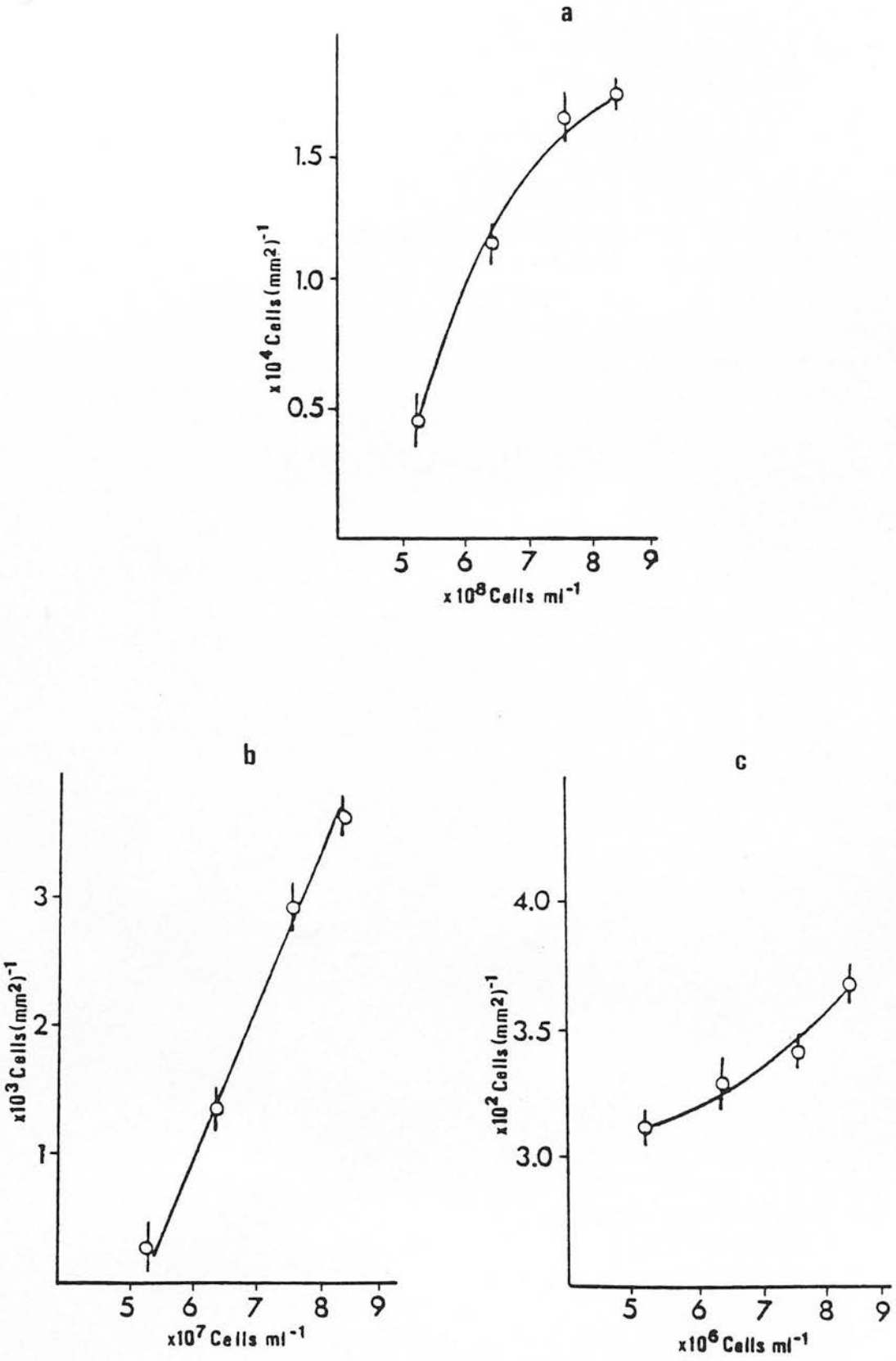


FIGURE 23:

The effect of culture concentration upon attachment of strain S61 to glass slides suspended in YE salts at 30°C, 120rpm. (a) = x 10^8 cells ml^{-1}, (b) = x 10^7 cells ml^{-1} and (c) = x 10^6 cells at 30°C, 120rpm.

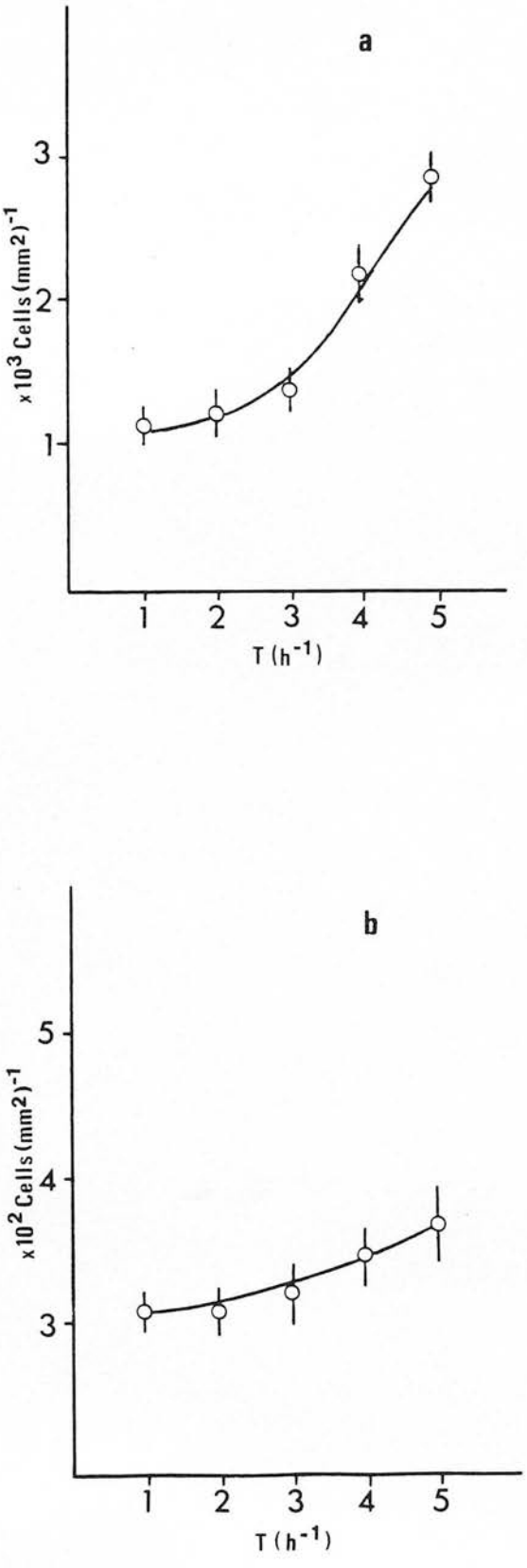


FIGURE 24: The effect of time upon attachment of Strain S61 at culture concentrations of (a) 6.3 x 10^7 cells ml^{-1} and (b) 6.3 x 10^6 cells ml^{-1}. Glass slides were suspended in YE salts at 30°C, 120rpm.

an increased number of collisons between the bacteria and substratum (Fletcher, 1977). This in turn offers a greater statistical opportunity for attachment to occur. Although the bacterial strain and the inert surface used are different to those used by Fletcher (1977), similar responses are shown. This may therefore indicate that the initial events in bacterial attachment are controlled by a nonbiological phenomena.

4.2.3 Effect of Temperature Upon Attachment

The temperature of the surrounding medium has previously been shown to influence bacterial attachment (Floodgate, 1972; Fletcher, 1977). In a series of experiments, Strain S61 was grown at either 9°C or 30°C, and assayed for levels of attachment at both of these temperatures (Fig 25). All culture concentrations were similar, 8.3×10^{7} cells ml^{-1}. Cells that were both grown and attached at 30°C showed an exponential increase in adhered numbers with time. In comparison, cells that were grown and permitted to attach at 9°C showed a different response. After 1h of immersion, the glass slide had a three fold increase in the number of attached cells. However, this number did not significantly change with time, fluctuating around the initial cell count. A possible explanation could be that at 30°C the cells are metabolically more active, colliding more often with the substratum. Hence, chemiadsorption is favoured. Cells that are grown at 30°C yet assayed for adherence at 9°C show a gradual increase in attachment with time. This result would support the concept of chemiadsorption. Cells that are grown at 30°C produce more EPS than at 9°C (Section 5.4). Consequently, if adhesion is mediated by specific cell surface receptors, growth at 30°C would leave fewer sites available to interact with the substratum. At 9°C growth however, polysaccharide does not saturate the surface receptors, hence the increased level of attachment over the initial 3h. Fletcher (1977) found that growth at a low temperature decreased the number of pseudomonad cells attached. In contrast, Floodgate (1972) observed the opposite using a motile gram negative rod of undetermined genus. Growth at 25°C as compared with 14°C and 4°C decreased the number of bacteria which settled. The appearance of an adhesion pad did not change with the temperature. These conflicting results may in part be due

FIGURES 25, 26 & 27:

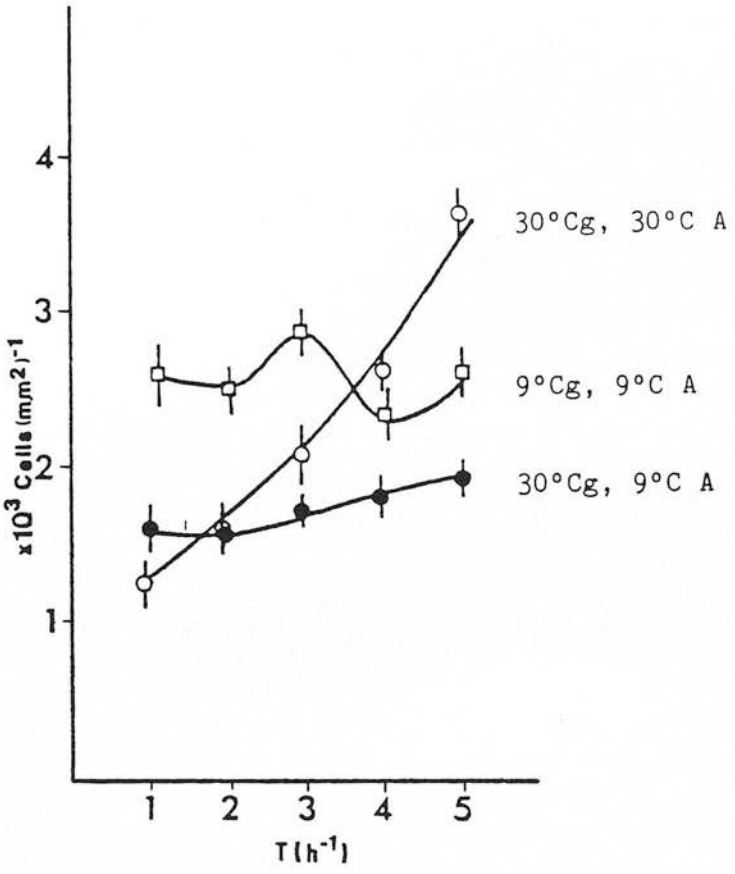


Figure 25: The effect of temperature upon growth and attachment strain S61 to glass slides. g= growth temperature, A= attachment temperature.

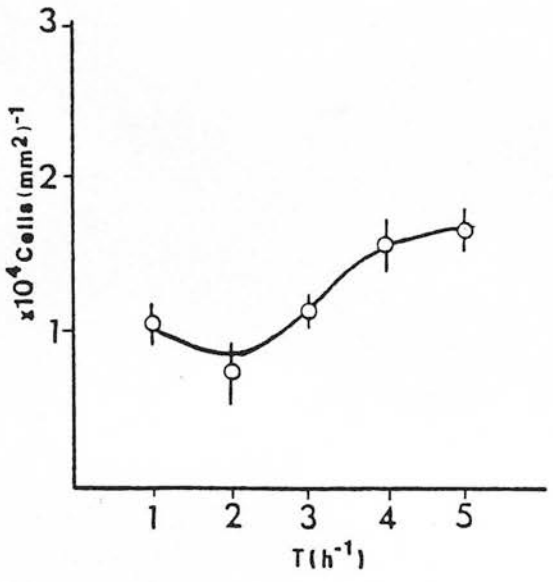


Figure 26: Attachment of strain S61 grown under a glucose limitation (0.065% w/v).

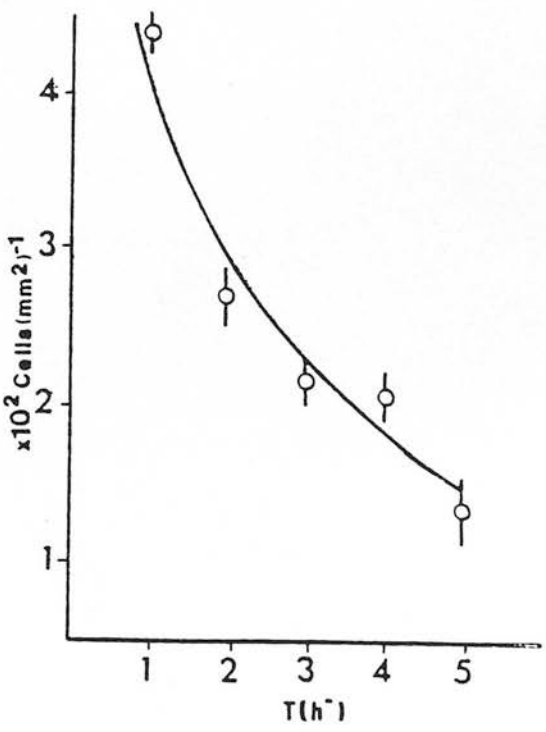


Figure 27: The effect of washing cells in 15mM EDTA upon attachment of strain S61.

to the type of organism used and the phase of the growth cycle it is in. Both Fletcher (1977) and Floodgate (1972) used marine bacteria, the isolate being in the stationary phase of growth in the latter's experiments. Adhesion is dependent upon the temperature of the medium, being a balance between the availability of cell surface receptors and chemiadsorption.

4.3 Cell Surface Effects

4.3.1 Glucose Limitation

The results from Section 3.5.2 indicate the possible involvement of specific cell surface receptors in the initial phase of bacterial adhesion. When grown under a carbon limitation, trace amounts of EPS are synthesized (Fig 13). SEM studies reveal the presence of surface microfibrils after 8h growth and polymeric strands after 16h (Section 6.3). The number of cells attached is slightly greater than the number adhering when grown with an excess of carbon; congo red staining reveals that the cells adhere mainly as individual units, with little association of polymeric material.

Strain S61 was grown under a glucose limitation, harvested, washed, and resuspended in YE salts to a final concentration of 2.4×10^7 cells ml^{-1}. Chemically clean slides were suspended in the culture and removed at regular intervals for cell counts. The results are illustrated in Fig 26.

Compared to cells (6.3×10^7 cells ml^{-1} culture concentration) grown in a carbon excess (Fig 24a) there is a five fold increase in the number attached when glucose limited. The rate of attachment is levelling off, indicating that the glass surface is becoming saturated. The result compares favourably with ideas proposed in Section 3.5.2. If the cell is producing only trace amounts of polymeric material, cell surface receptors will be available for adhesion in greater numbers. Under conditions of carbon excess, EPS are produced in quantities that are enough to saturate possible receptors. These results are similar to the findings of Brown *et al.*, (1977). Inert surfaces confer an ecological advantage to the attached micro-organisms. The exposed surface serves as an area for the concentration of limiting nutrients, making them more readily available to the attached population than to free-living cell types (Dawson *et al.*, 1981; Kjelleberg *et al.*, 1982).

4.3.2 EDTA Wash

A further indication of the possible involvement of a specific cell surface receptor is provided in the results graphically illustrated in Fig 27. Log phase cells (S61) were harvested and washed in YE salts containing 15mM EDTA (disodium salt), resuspended to a final concentration of 6.2×10^7 cells ml^{-1} and assayed for adherence to glass slides as previously described. After 1h, there is a ten fold decrease in the number of attached cells compared to cells untreated with EDTA (Fig 24a). With time, an exponential decrease in adhesion is observed; ie. detachment occurs. EDTA is a chelating agent, known to sequester Mg^{2+} ions preferentially and to disrupt outer membranes in gram negative bacteria. Mg^{2+} ions forming ionic interactions between the LPS molecules in the outer membrane are extracted by the salt. This results in a weakening of LPS-protein interactions, causing permeability at the cell surface. Other detrimental effects caused by EDTA include loss of ribosomes and reduction in the RNA content of the cell. Disrupting the outer membrane, thereby causing cell detachment would suggest that the surface component mediating adhesion is closely associated with the cells outer layer. This is likely to have proteinaceous characteristics.

4.3.3 Attachment of S61 Mutants

Mutants altered in cell surface characteristics were isolated from Strain S61. NTG mutagenesis was used to produce a non-mucoid varient, NS61-20b, (Plate 2b) and uranyl nitrate to enrich for a novobiocin resistant mutant, US61(iii). The growth characteristics of mutant NS61-20b have been previously described (Section 2.2.3). Initially, selection was based solely on the morphological appearance of the colonies. However, subsequent growth studies showed low levels of EPS production compared to the wild type.

The attachment ability of NS61-20b is illustrated in Figure 28. Using a culture concentration of 5.2×10^7 cells ml^{-1}, a similar level of attachment occurred as was observed for the mucoid parent cell. The slight reduction in number of adherent cells is not significant, and is probably due to a lower culture concentration. This result compliments the observations made in Section 3.3. Congo red staining revealed non-mucoid cells adhering in equal numbers to the mucoid type, but without the production or association

FIGURES 28 & 29:

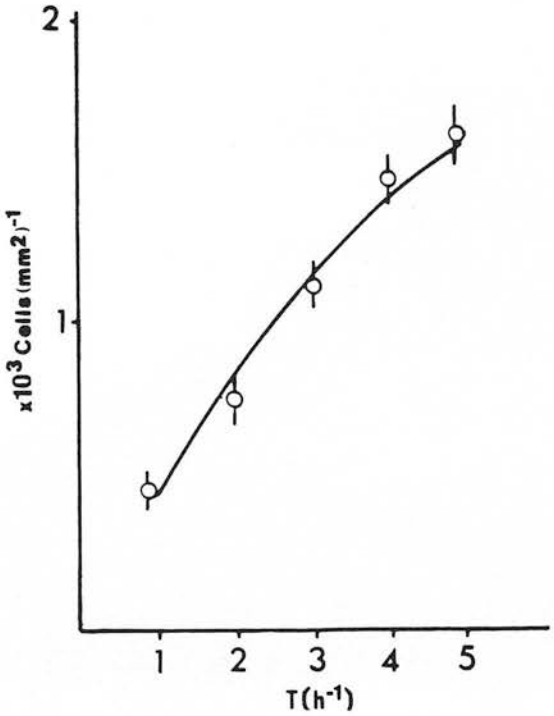


Figure 28: Attachment of strain NS61-20b, a non-mucoid mutant, to glass slides suspended in YE salts.

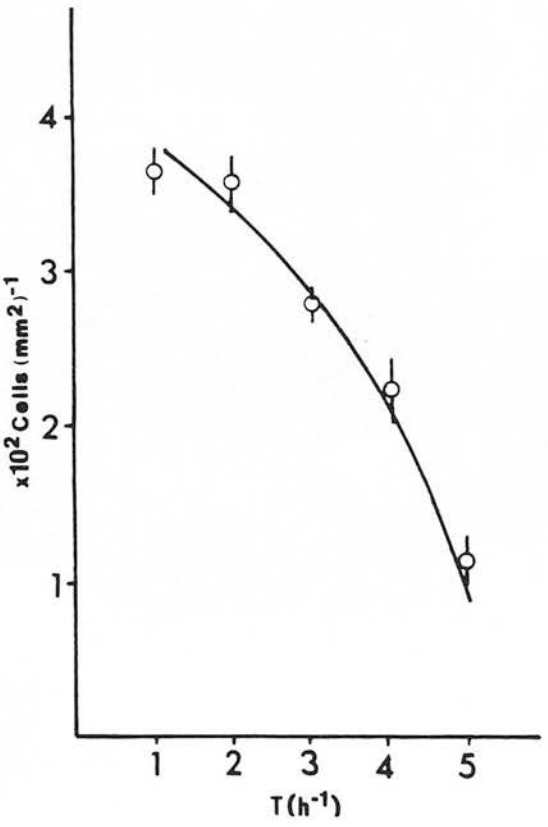


Figure 29: Attachment of strain US61 (iii), a novobiocin resistant mutant, to glass slides suspended in YE salts.

of polymeric material. Microcolony formation was not observed. Pringle et al., (1983) described similar results. A freshwater strain of Pseudomonas fluorescens produced a mucoid mutant which showed lower levels of attachment than the non-mucoid wild type. No major differences in the outer membrane profiles of both mutant and wild type could be detected. These results strengthen the proposal that EPS synthesis is not a prerequisite for bacterial adhesion. The polymeric material may not participate in the initial phase of attachment as was previously suggested (Corpe, 1970b; Costerton et al., 1978) but in the development of subsequent bacterial film.

The response of mutant US61(iii) is shown in Figure 29. As with the EDTA washed cells, there was a lower level of adhesion initially, followed by a rapid detachment of cells. Novobiocin acts on the cell surface, leading to the accumulation of UDP-N-acetyl muramic acid peptides. DNA,RNA,LPS and proteins can also be affected. The minimum inhibitory concentration calculated for Strain S61 was between 0.625 and 0.312 $mgml^{-1}$; US61(iii) was resistant to values between 0.625 and 1.100$mgml^{-1}$. Novobiocin has been used to effect in the selection of mutants with altered cell surface components, particularly lipopolysaccharides. Because of the complex mode of action, it is not possible from these results alone to state with any detail the site of novobiocin attack causing detachment. Suffice to say an alteration in the cell surface has occurred.

SECTION 5 Continuous Culture Studies

5.1 Introduction

Biofilms can develop on any surface exposed to a growing microbial culture. Within a chemostat, problems can be created by colonization of both fermenter walls and reactor assembly components. These include variable continuous culture washout, atypical ecological niches within the reactor and erratic effulent biomass concentrations (Bryers, 1984). The effects of biofilm formation on fermenter operation have been studied in detail (Topiwala & Hamer, 1971; Wilkinson & Hamer, 1974; Bryers & Characklis, 1982) since the earlier considerations of both Larsen and Dimmick (1964) and Munson and Bridges (1964). A particular advantage of continuous culture is that attachment and growth of cultures may be studied over an extended time period. This is not possible using batch culture, the conditions changing continuously. With a chemostat culture, both the bacterial growth rate and the growth environment can be independently varied between wide limits. Under most circumstances, the dilution rate of the chemostat is equivalent to the growth rate of the cells.

Continuous culture has been used for a number of studies involving adherent micro-organisms. Brown et al., (1977) and Wardell et al., (1980) reported the effect of varying the nature of the limiting substrate upon colonization of suspended surfaces by bacteria enriched from freshwater. Hendricks (1974) investigated the difference in metabolic activity between sorbed and suspended populations, and the effect of varying cultural parameters (carbon source, pH, oxygen tension) upon biofilm accumulation of Pseudomonas putida was studied by Molin and Nilsson (1983). Very little attention however, has been paid to the physiological condition of the organisms concerned. Pringle et al., (1983) used continuous culture methods of isolate Pseudomonas fluorescens mutants differing in cell surface characteristics from the original organism. These were subsequently grown in batch culture and compared both structurally and compositionally to the wild type. Physiological studies of organisms isolated from a natural environment are best achieved under conditions that are both chemically and physically invarient

with time. Application of continuous culture techniques can allow, for example, the influence of environmental parameters to be studied with greater accuracy.

5.2 Chemostat 1 (C-1): Effect of Low Salt Concentration.

Analysis of the river water by atomic absorption spectroscopy revealed the low concentration of ions present (Section 1.2). A New-Brunswick bench top chemostat (working volume 310ml) was used to study the effect on the physiology of the organisms at different dilution rates using a growth medium with a low ionic content, as indicated (Table 22).

Casamino Acids	0.1 gL^{-1}
Yeast Extract	0.1 gL^{-1}
YE Salts	10ml
Glucose	10.0 gL^{-1}
dH_2O	to 1 Litre

TABLE 22: Composition of growth medium for chemostat C-1.

A culture of Strain S61 was grown in batch (100ml in 250ml flask) and was used to inoculate the fermenter (40ml). Nutrient flow was started to affect a dilution rate (D) of 0.010h^{-1} for 8h, allowing the cells to adapt to chemostat conditions. Thereafter, steady state conditions were achieved for each D value before harvesting and analysis of material. Growth temperature was 30°C.

The results for polymer yield, cell numbers, cell dry weight and culture pH with varying dilution rate are illustrated in Figure 30A. As the dilution rate increases, the number of viable cells drops steadily from 8 x 10^8 cells ml^{-1} to about 2 x 10^8 cells ml^{-1}. Cell dry weight is also observed to drop with an increase in D, but to a lesser extent. Values appear to stabilize after D=0.06h^{-1}, indicating the possibility of a storage compound (such as B-hydroxy-butyric acid) being synthesized. No observable difference in cell size was noted. EPS production appears to decrease with increasing dilution rate. However, calculations revealing the amount of polymer produced by 1mg of cells (dry weight) (Table 23) show that there is a net increase in polymer production with dilution rate, except for a D value of 0.13h^{-1}. Analysis of the polymer and culture

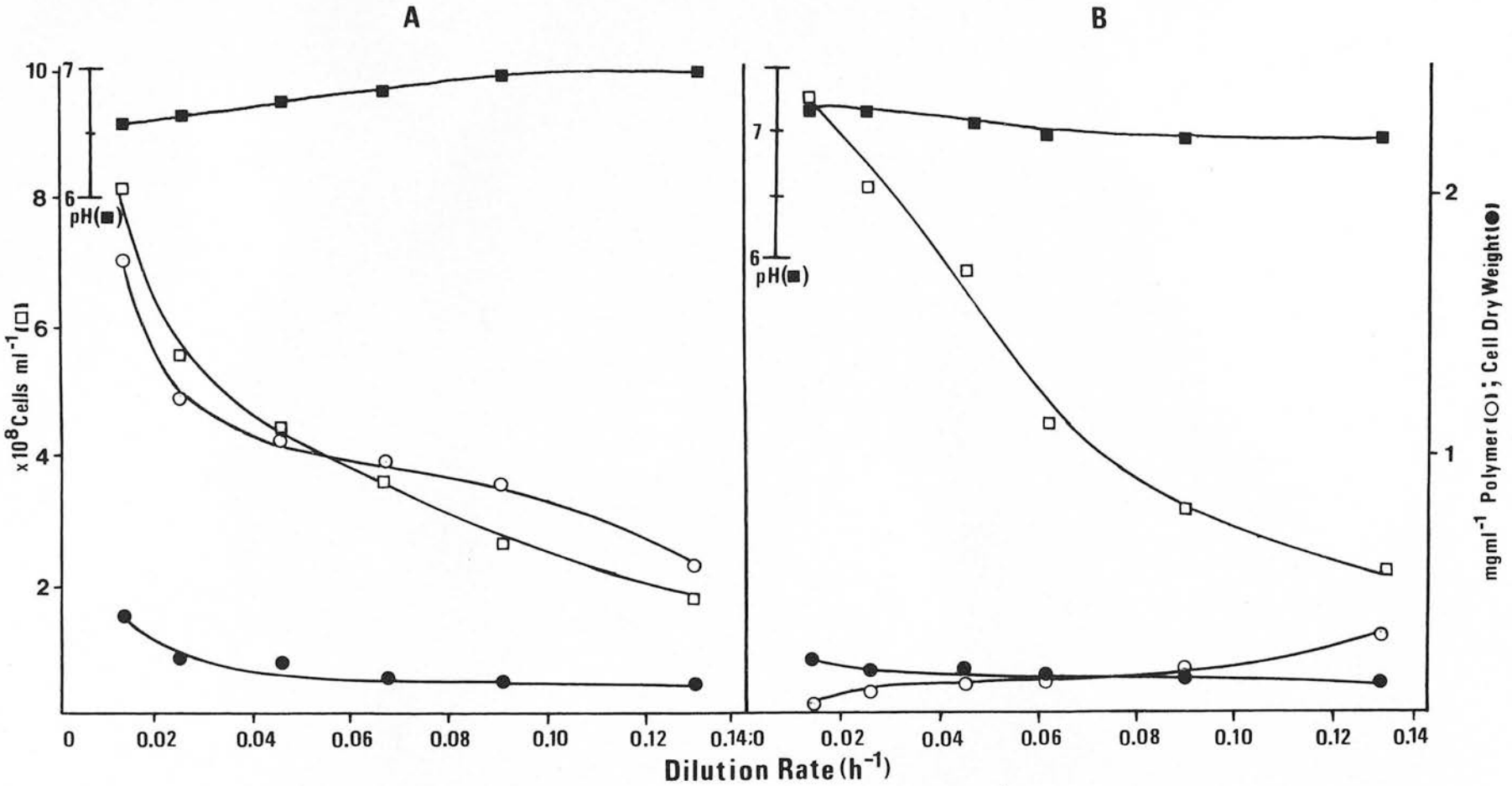


FIGURE 30: Growth of strain S61 in continuous culture under (A) Low Salt Concentration and (B) Carbon Limitation. Polymer production(O); cell dry weight (●); viable cell number (□) and pH (■) as a function of dilution rate. 30°C, impellor 400rpm.

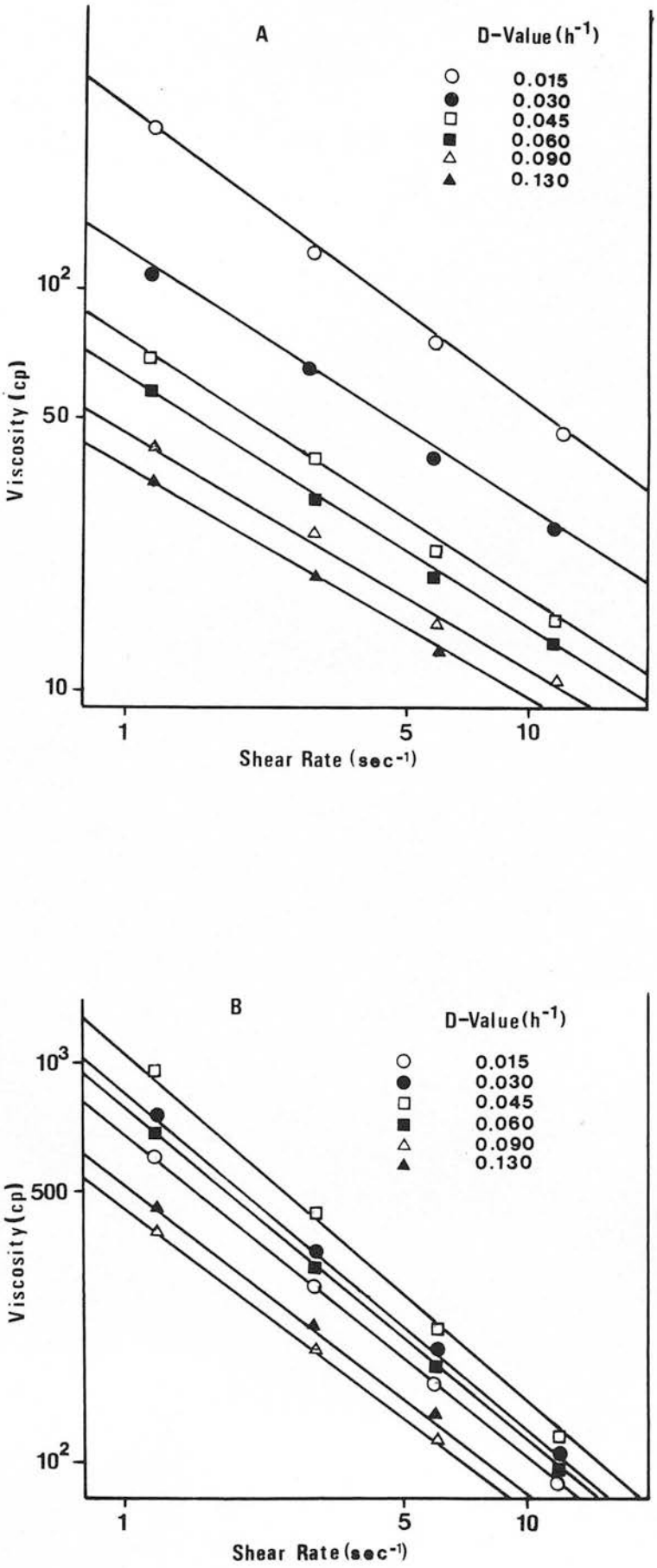


FIGURE 31: Viscosity of (A) Fermentor culture and (B) 0.1% Polysaccharide solutions as a function of dilution rate. Growth of strain S61 under low salt concentration, 30°C, impellor 400rpm.

viscosities (Fig 31A,B) show that culture viscosity decreases with increasing D, whereas there is no observable pattern with polysaccharide viscosity. Thus, as the dilution rate increases, more polymer is produced (until a specific D value) with a subsequent decrease in culture viscosity. The results may be similar to those when observing polymer production in the absence of Ca^{2+} and Mg^{2+} ions (Section 2.2.6). When the ionic content of the medium (which includes both Ca^{2+} and Mg^{2+} ions) is reduced to a low level, regulation of polysaccharide synthesis may be affected.

Total carbohydrate estimations (Dubois et al., 1956) revealed a similar trend to the above results. Chemical analysis of the polymers indicated no significant change in either acetate or pyruvate content with dilution rate. Acetate varied from between 17.8% at the lowest dilution rate, to 12.7% at $D=0.13h^{-1}$. However, this was deemed not significant as it fell within experimental error. Similarly, pH measurements only increase slightly with increasing D.

Outer membrane proteins were harvested as previously described, and examined by SDS polyacrylamide gel electrophoresis. Due to insufficient material, the gel was not suitable for photography; Fig 32 represents the protein bands observed. A noticeable effect is the increase in amounts of most of the major proteins. This is further qualified by a densitometry scan of the gel (Fig 33). The higher molecular weight proteins appear to increase more substantially than any of the other proteins. This includes the cell surface receptors isolated in Section 7.3. There is however, no addition or deletion of any protein from the outer membrane profile.

At high dilution rates, the increased production of specific outer membrane proteins and greater levels of polysaccharide synthesis may be of ecological significance. Organisms would have a greater chance to adhere to inert surfaces where conditions are nutritionally more favourable.

5.3 Chemostat 2 (C-2): Effect of Glucose Limitation.

Previous results (Sections 2, 3 4) indicate that low levels of polysaccharide systhesis occur under glucose limiting conditions, yet increased cell adhesion occurs. The effect of glucose limitation on the cell surface was studied at varying dilution rates. Batch

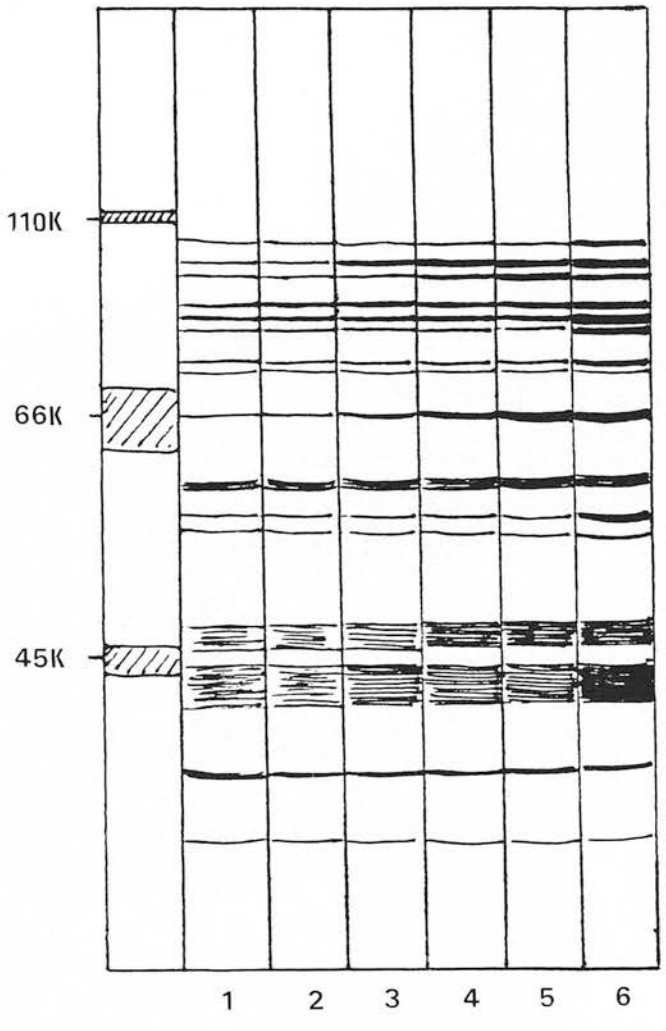
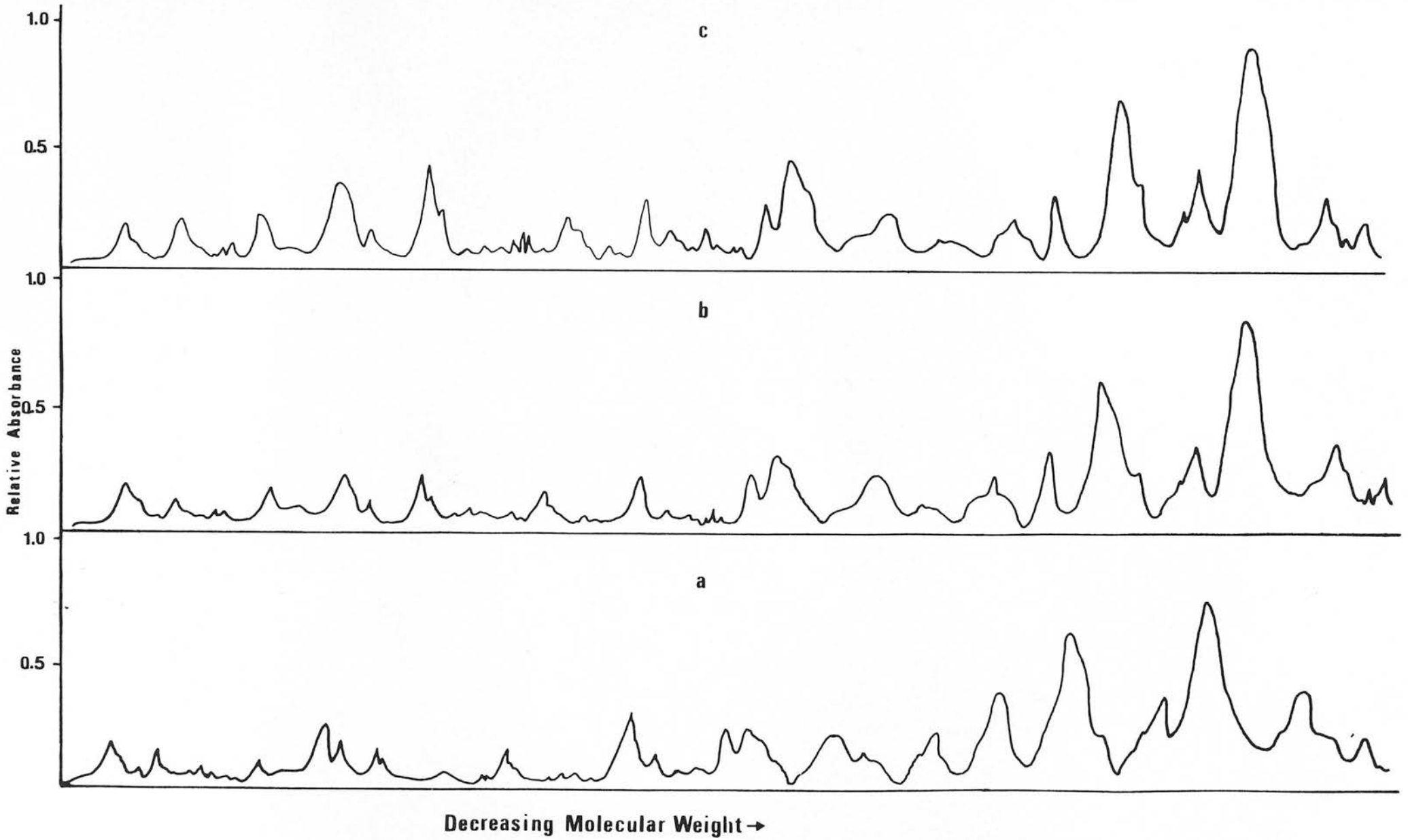
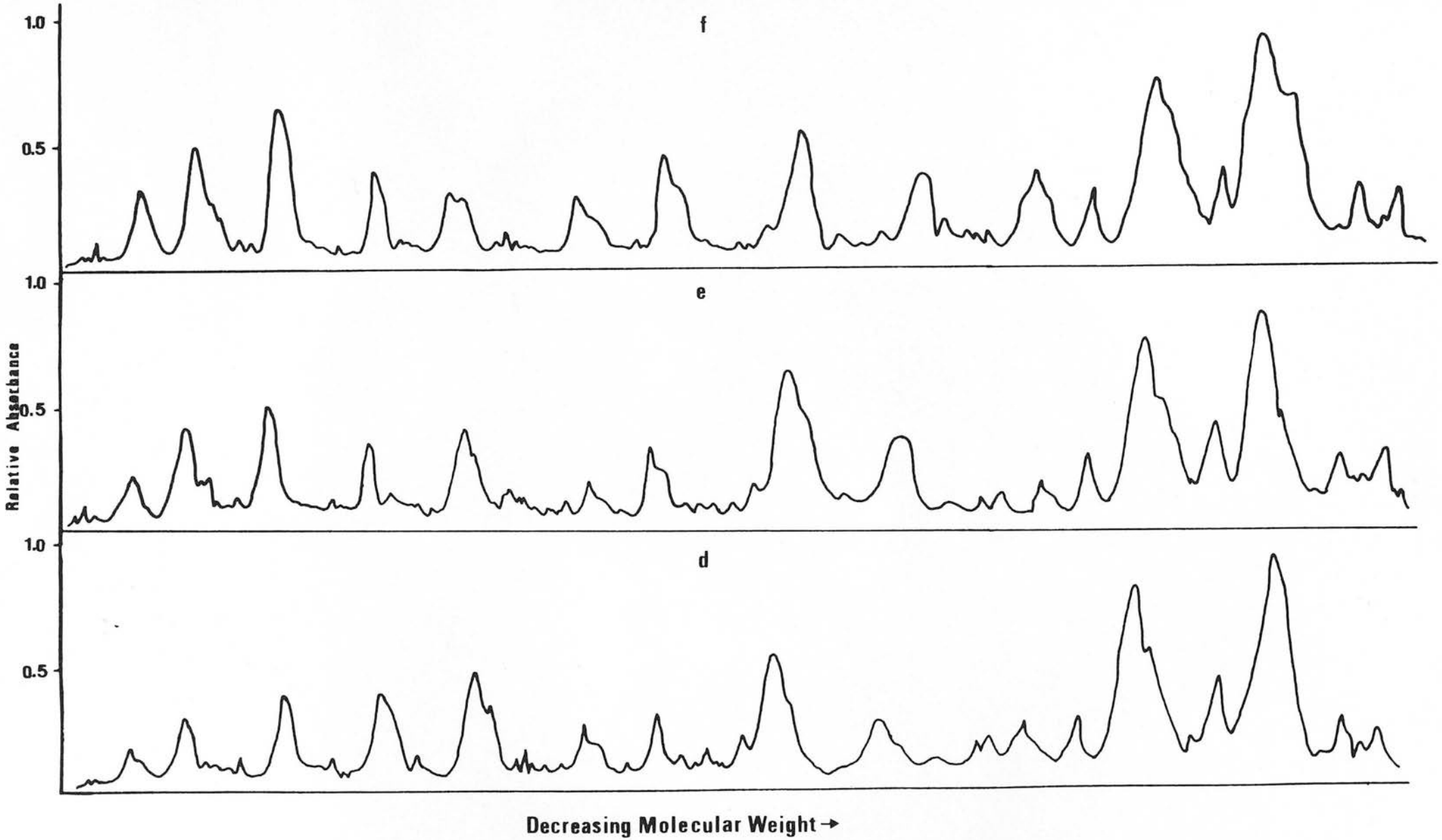


FIGURE 32: Schematic representation of SDS-PAGE outer membrane protein profile for strain S61 grown in continuous culture under low salt concentration. Material harvested at dilution rates (1) $0.015h^{-1}$; (2) $0.030h^{-1}$; (3) $0.045h^{-1}$; (4) $0.060h^{-1}$; (5) $0.090h^{-1}$ and (6) $0.130h^{-1}$. Protein standards are indicated.

FIGURE 33: Densitometer scan of outer membrane proteins harvested from strain S61 grown under low salt concentration in continuous culture. Samples harvested at dilution rates (a) $0.015h^{-1}$; (b) $0.030h^{-1}$; (c) $0.045h^{-1}$; (d) $0.060h^{-1}$; (e) $0.090h^{-1}$ and (f) $0.130h^{-1}$.


1.0
0.5
c
1.0
b
0.5
1.0
a
0.5
Relative Absorbance
Decreasing Molecular Weight →


f
e
d
1.0
0.5
Relative Absorbance
Decreasing Molecular Weight →

culture experiments were used to determine the limiting concentration of glucose. The medium adopted was 1/10 YE supplemented with 0.065% (w/v) glucose. All other growth conditions were as before.

A similar response to increasing the dilution rate was observed for the viable cell count (Fig 30B); a steady decrease with increasing D value. Cell dry weight also showed a similar response, dropping gradually as the dilution rate increased. Polymer production however, showed an increase with D. Again, this was reflected by the total carbohydrate measurements (not shown). The amount of polymer (dry weight) synthesized per mg. (dry weight) of cells reveals that there is a steady increase in polymer production with D (Table 23), the largest increase being between D values of 0.09 and $0.13h^{-1}$. Thus, under glucose limitation, Strain 61 does produce polysaccharide, though in reduced amounts compared to growth with a glucose excess. Viscosity measurements of the polymer (Fig 34) revealed an increase in viscosity with an increase in dilution rate. The viscosity of the culture did not significantly vary with the dilution rate (results not shown); acetate and pyruvate content of the polymer showed no significant change with dilution rate. Culture pH values remained almost constant (pH 7.15) at the various dilution rates.

Slight changes were observed in the outer membrane profile (Plate 10). Notably, the high molecular weight proteins were shown to increase with increased growth rate. What relationship this finding bears to the observations that greater adhesion occurs by microfibrils (Section 6.3) is as yet unknown.

5.4 Chemostat 3 (C-3): Effect of Lowered Temperature

Bacterial adhesion has been shown to be reduced at low temperatures (Fletcher, 1977; Pedersen, 1982). Growth of Strain S61 was carried out at the temperature of original isolation viz 9°C. YE medium supplemented with 1% (w/v) glucose was used to support growth, all other conditions being similar to those previously described.

A steady decrease in cell numbers is again observed with increased growth rate (Fig 35). However, compared to the previous chemostat conditions, there is a ten fold reduction in viable cell numbers. Similarly, the doubling time was increased approximately

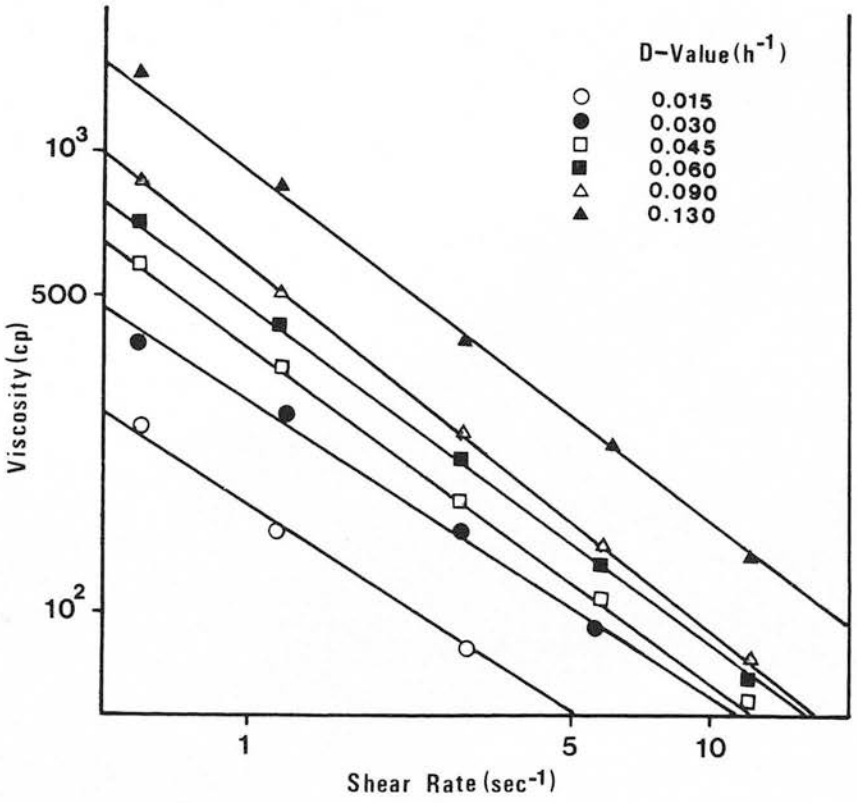


FIGURE 34: Viscosity of 0.1% Polysaccharide solutions as a function of dilution rate. Growth of strain S61 glucose limitation, 30°C, impellor 400rpm.

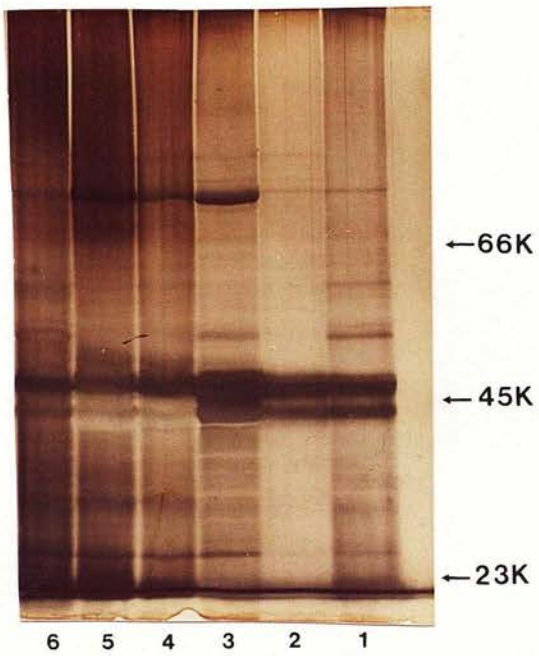


PLATE 10 Gel electrophoresis of outer membrane proteins isolated from strain S61 grown under carbon limitation in continuous culture. Tracks 1-6 correspond to dilution rates (1) $0.015h^{-1}$; (2) $0.030h^{-1}$; (3) $0.045h^{-1}$; (4) $0.060h^{-1}$. Molecular weight markers indicated.

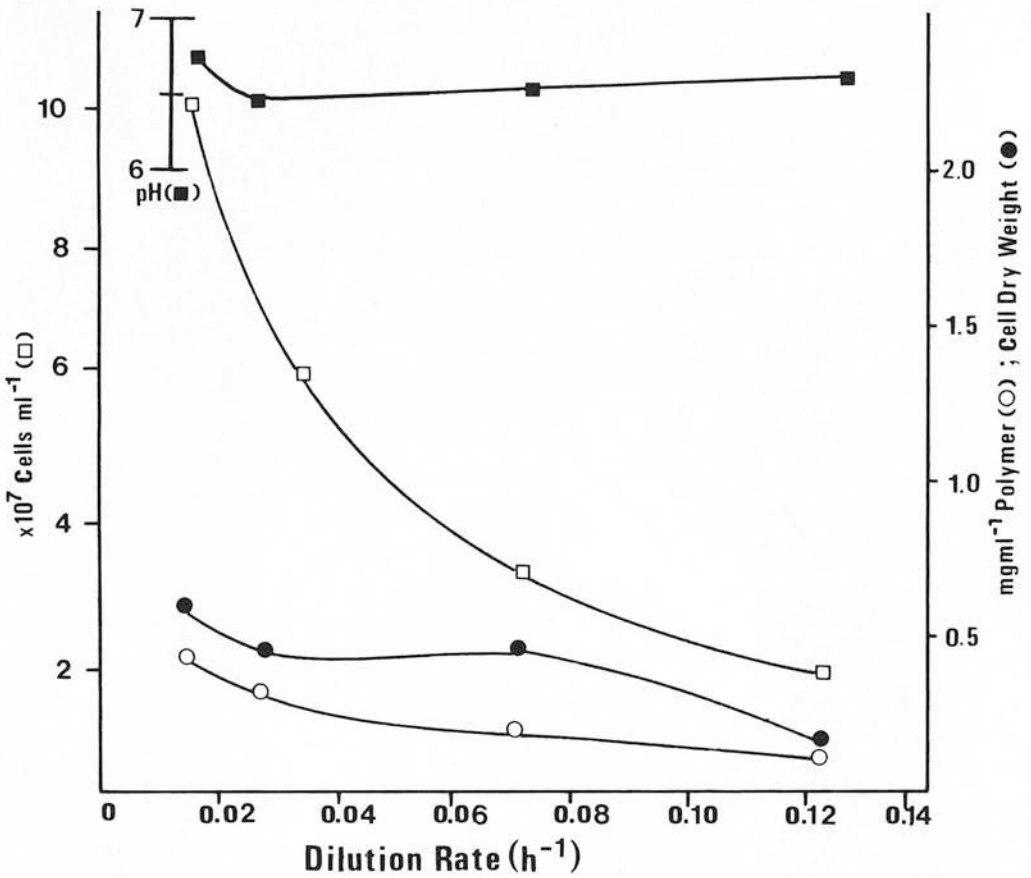


FIGURE 35: Growth of strain S61 in Continuous Culture at 9°C. Polymer production (○); cell dry weight (●); viable cell number (□) and pH (■) as a function of dilution rate. Impellor, 400rpm.

five fold. Both polymer yield and cell dry weight were observed to decrease with dilution rate, this result being reflected by the results in Table 23. As dilution rate increases, the amount of polymer produced per mg cells (dry weight) decreases. The viscosity of the culture medium was low, but did not significantly vary with D (results not shown). Polymer viscosity was also low compared to the previous chemostat studies, and decreased with increased growth rate (Fig 36). Thus, as the dilution rate increased, polymer production and viscosity decreased. As previously, no differences were observed in the chemical composition of the polymers. pH values varied between 6.8 and 6.5

Molin and Nilsson (1983) reported that decreasing the temperature of growth had no effect on biofilm formation, but did influence the size of the suspended cells. As the temperature decreased, cell size increased. However, no such effect was observed to occur in the system tested here. Reports in the literature on the effect of temperature are contradictory. Trueba et al., (1982) indicated that cell size decreased with decreasing temperature, whereas Shehata and Marr (1975) argued that there is little difference in cell size. The differences in results are most probably due to the different organisms used and the fact that in some cases the temperature effect was tested under different growth rates.

The outer membrane profile for Strain S61 grown at varying dilution rates at 9°C is illustrated in Plate 11. Differences in the amounts of proteins can be observed, both from the photograph (Plate11) and from the densitometry scan (Fig 37). No particular pattern appears to be occurring. Different proteins increase in amount at different growth rates. Temperature would thus appear to play a significant role in the composition of the outer membrane.

The overall results for the three chemostat experiments would indicate that the cell surface is susceptible to change by varying parameters and growth rates. Polysaccharide production generally increased with increasing dilution rate (ie. under low ion concentration and glucose limitation), yet decreased at a low temperature. Rudd et al., (1982) showed that polymer production by Klebsiella aerogenes at 25°C was greater in continuous culture than in batch culture, and increased as the dilution rate decreased.

	D (h^{-1})					
	0.015	0.030	0.045	0.060	0.090	0.130
C-1	4.50	5.30	5.70	6.50	6.70	4.80
C-2	0.41	0.52	0.60	0.86	1.06	2.06
C-3	0.82	0.78	nd	0.66	nd	0.59

TABLE 23: Polysaccharide production (mg dry weight) per mg (dry weight) cells. Chemostat conditions (C-1) Low Ionic Composition, (C-2) Glucose Limitation and (C-3) Low Temperature. nd = not done

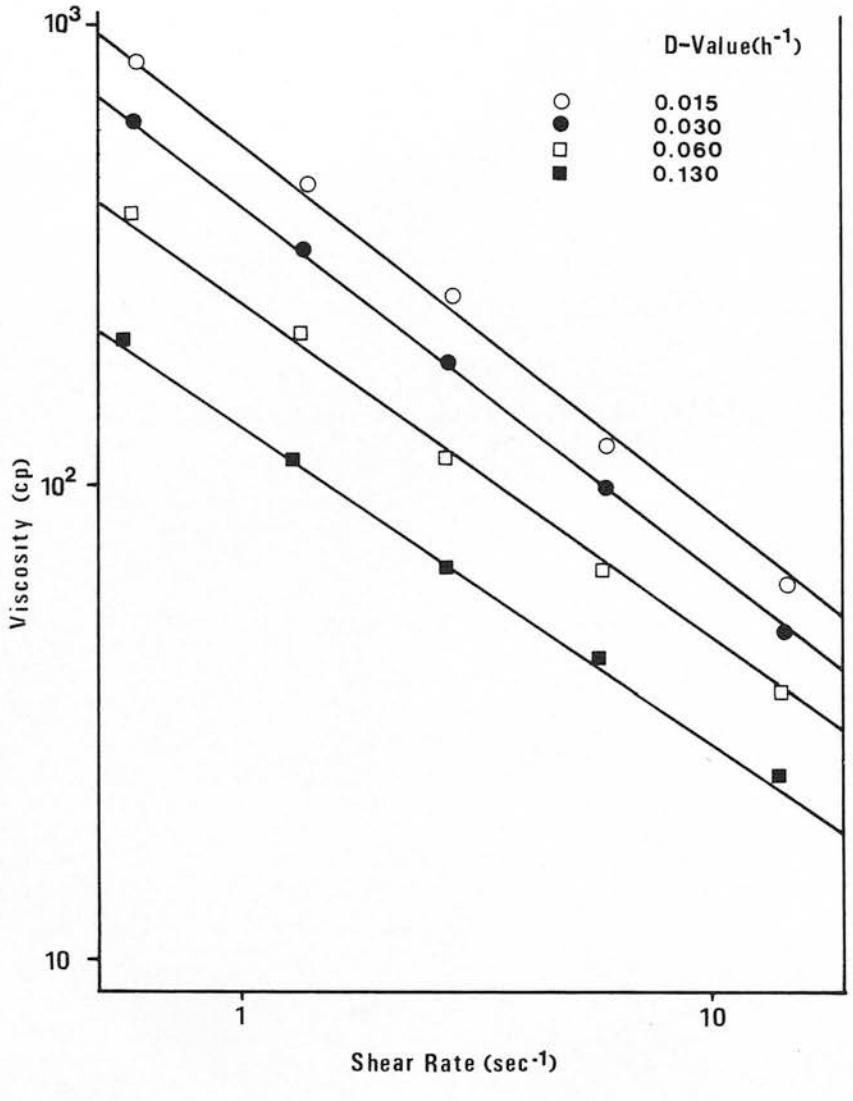


FIGURE 36: Viscosity of 0.1% Polysaccharide solutions as a function of dilution rate. Growth of strain S61 at 9°C, impellor 400rpm.

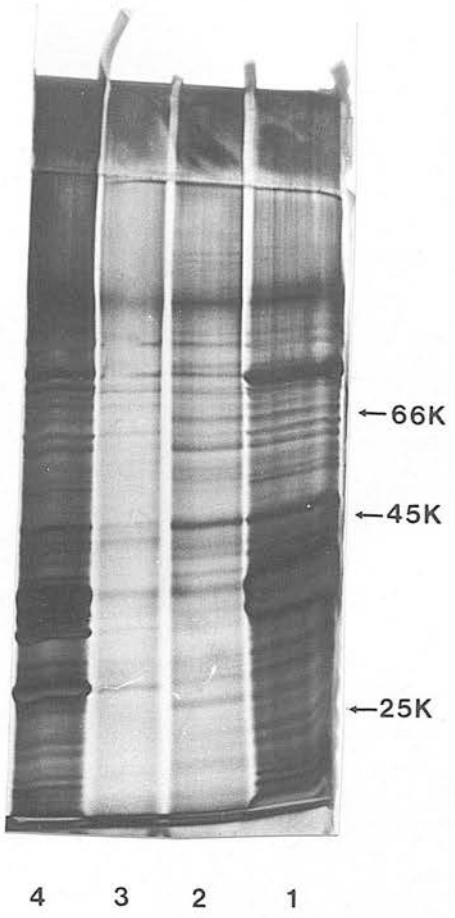
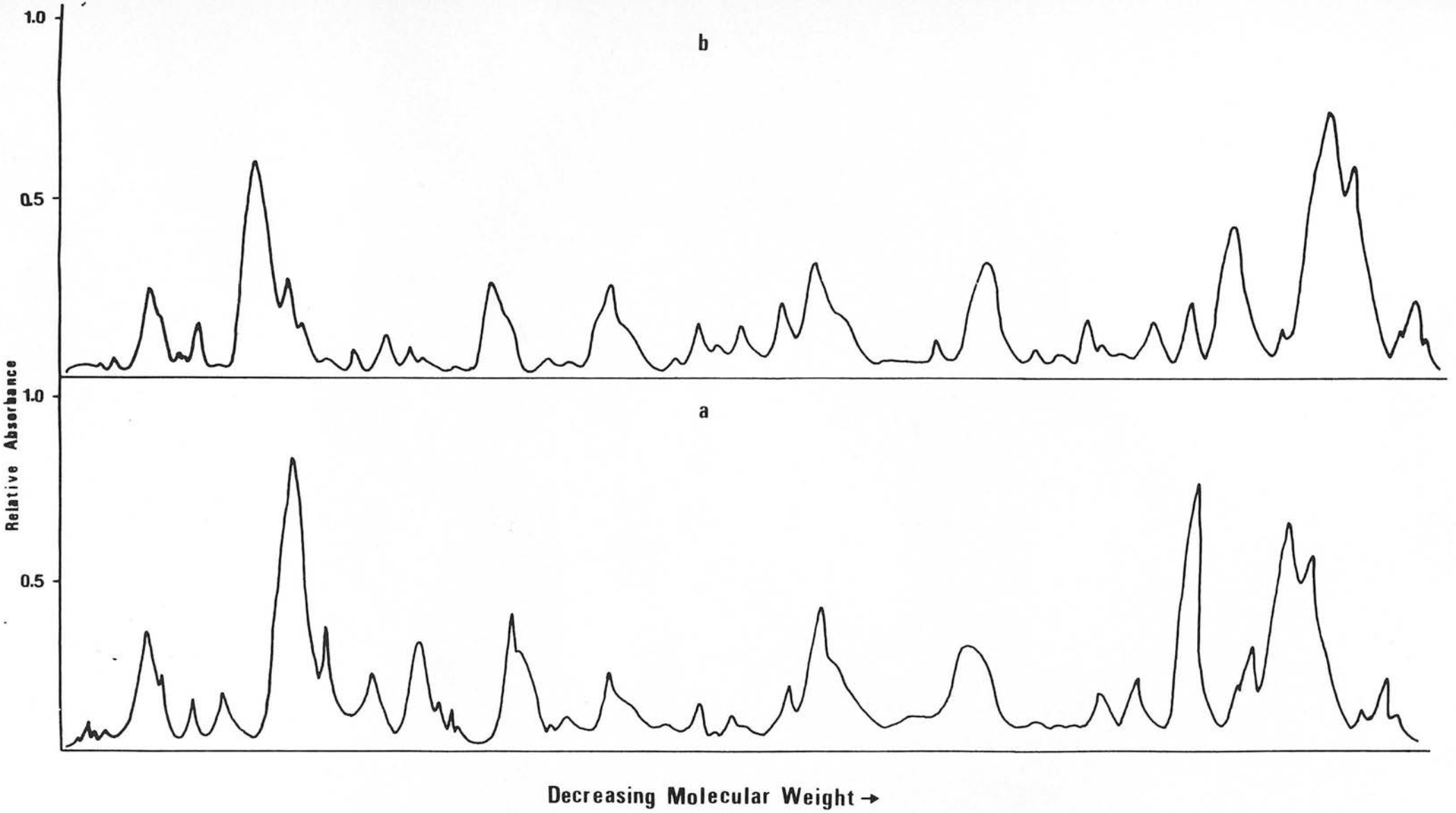
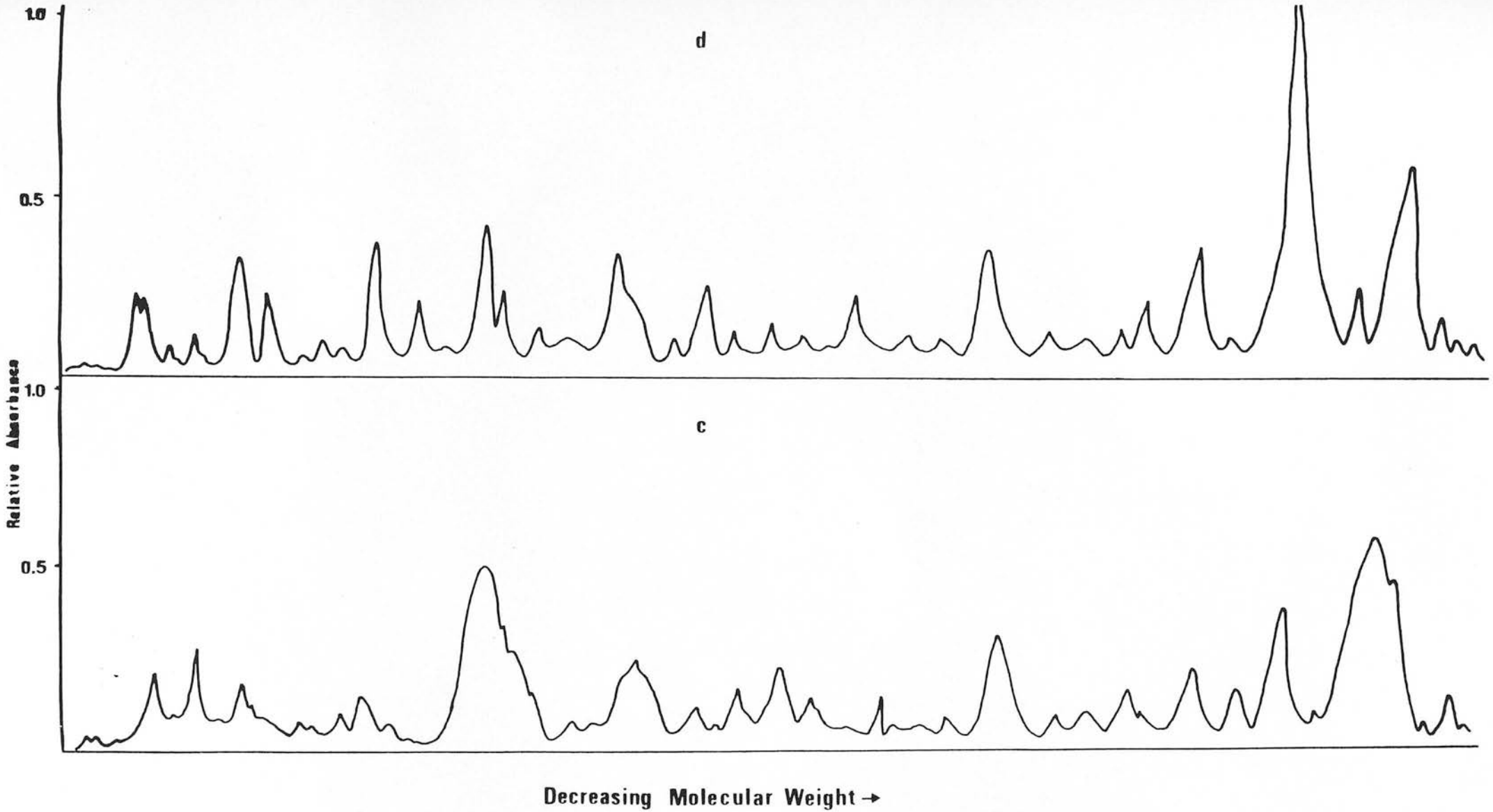


PLATE 11: Gel electrophoresis of outer membrane proteins isolated from strain S61 grown at 9°C in continuous culture. Material harvested at dilution rates (1) $0.015h^{-1}$; (2) $0.030h^{-1}$; (3) $0.060h^{-1}$ and (4) $0.130h^{-1}$. Molecular weight markers indicated.

FIGURE 37: Densitometer scan of outer membrane proteins harvested from strain S61 grown at 9°C in continuous culture. Dilution rates sampled were (a) $0.015h^{-1}$; (b) $0.030h^{-1}$; (c) $0.060h^{-1}$ and (d) $0.130h^{-1}$.


1.0
0.5
b
1.0
a
0.5
Relative Absorbance
Decreasing Molecular Weight →


1.0
0.5
d
1.0
c
0.5
Relative Absorbance
Decreasing Molecular Weight →

Differences in polymer viscosity appear to be related to the different growth parameters. No change from capsulation to solely slime producing cells was observed as the dilution rate decreased. In the natural environment, low yields of polysaccharide would be assumed to be produced. Therefore, a selective advantage would exist with micro-organisms that could produce enough material to allow adhesion to occur on a permanent basis at high D values on nutritionally rich surfaces. It could be argued however, that at low D values it would be more favourable to have a high-EPS-yielding organism to allow the cells to compete with other cells for nutrient accumulation.

Outer membrane proteins generally increase in amount with an increased growth rate, particularly the higher molecular weight proteins. This is not the case however at low temperatures, where a varied response is observed. Changing both the growth rates, and the culture conditions markedly influence the cell surface. Tempest and Ellwood (1969) observed that changing the growth rate in either a glycerol or Mg^{2+} limited chemostat influenced the composition of the cell wall in Aerobacter aerogenes. Both the KDO and heptose components of the lipopolysaccharide varied with the dilution rate.

A disadvantage with the studies reported here, is the fact that the adhesive ability of the organism tested (S61) was not assayed. A complete picture could then have been composed comparing changes in the cell surface with the cells ability to attach to inert surfaces.

SECTION 6 Scanning E.M. Studies

6.1 Introduction

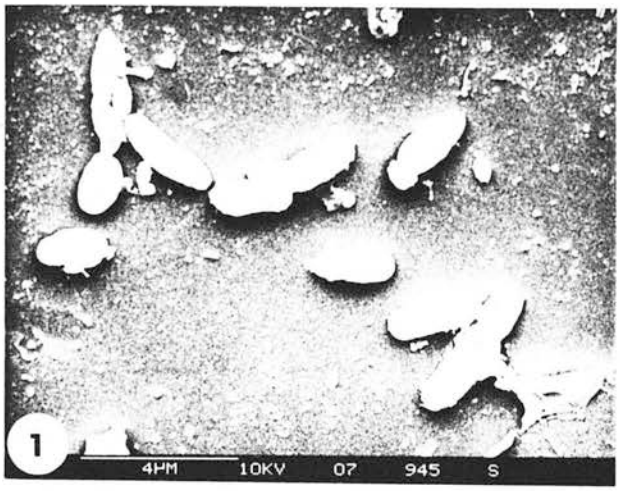
The use of Scanning E.M. reveals details of bacterial attachment which would otherwise be impossible to observe. Limited resolution of the light microscope, and the need for ultrathin sections in the Transmission E.M. discount these methods for a detailed examination of biofilm formation. Attached bacteria can be related to the topography of the test surface, the fine extracellular polymers mediating their adsorption being clearly demonstrated. Advantages of SEM include a large depth of focus (cf 300 fold greater than light microscopy), a greater degree of magnification (20-25,000 fold increase) and a resolution down to 25nm. No embedding or sectioning of material is needed, reducing the possibility of artifacts arising during sample preparation. Most importantly, bacteria can be observed adhering naturally to a variety of surfaces, either in laboratory experiments or in situ.

Previous results (Section 3,4) have suggested that EPS are involved in microcolony formation and do not play a role in the initial adhesive mechanism. When grown in YE media with a 1% carbon source, isolate S61 is observed to be attached to the surface and associated with carbohydrate containing material. With time, microcolonies form and increase in size, always with a corresponding increase in polysaccharide like material. However, when grown under a glucose limitation, strain S61 adheres in slightly greater numbers without the formation of microcolonies. Little in the way of polysaccharide material is observed associated with the attached cells. The aim of this section is to look at the cell surface involvement of Strain S61 adhering under both a glucose excess (1% w/v) and limitation (0.065% w/v). As a comparison, the cell surface from a sample of the planktonic population will also be examined.

6.2 Attachment of S61: Carbon Excess.

The results for S61 grown in YE media supplemented with 1% glucose are illustrated in Plate 12. After 8h growth, polymeric material can be observed on the cell surface. This is in the form of stout strands and appears to be continuous with the cell surface. Taking into account the increase in magnification from the light microscope, the cells are not yet forming microcolonies.

PLATE 12: SEM micrographs of Strain S61 adhering to glass coverslips suspended in YE media supplemented with 1% glucose. Coverslips removed after 8h (1,2), 16h (3,4) and 24h (5,6).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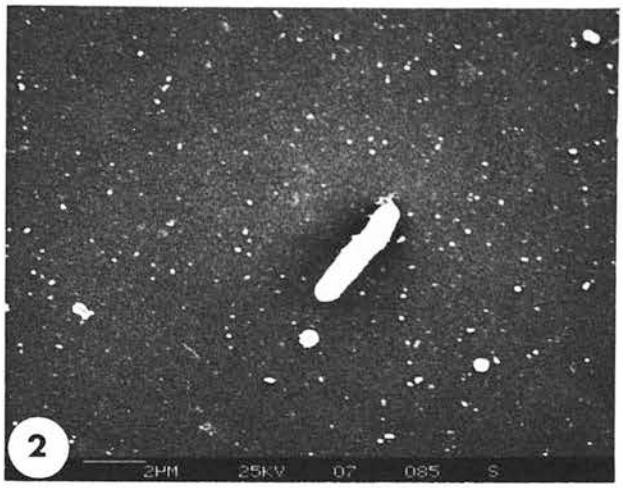
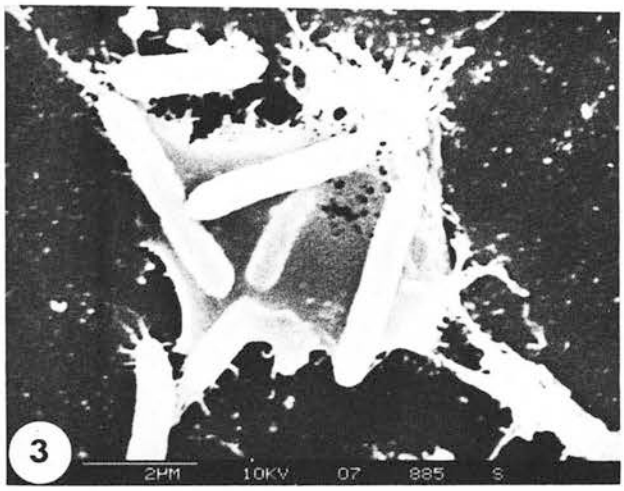

2
2PM 25KV 07 085 S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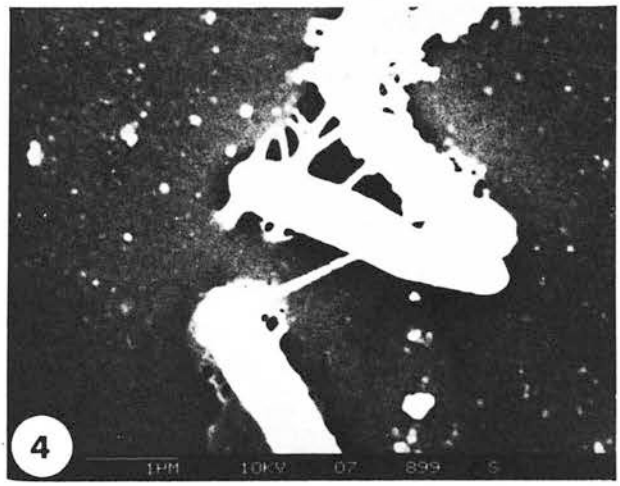
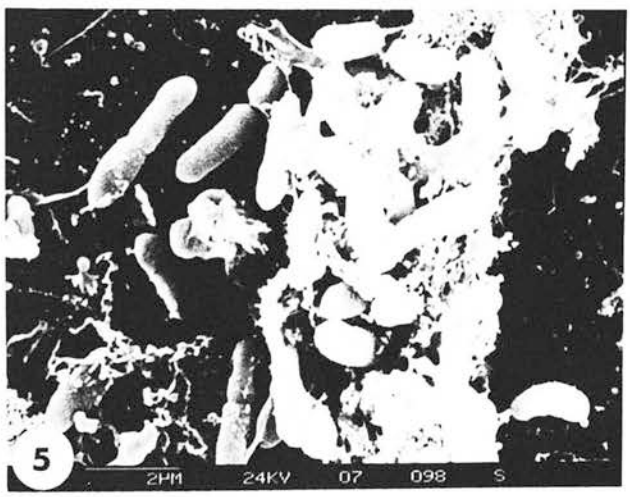
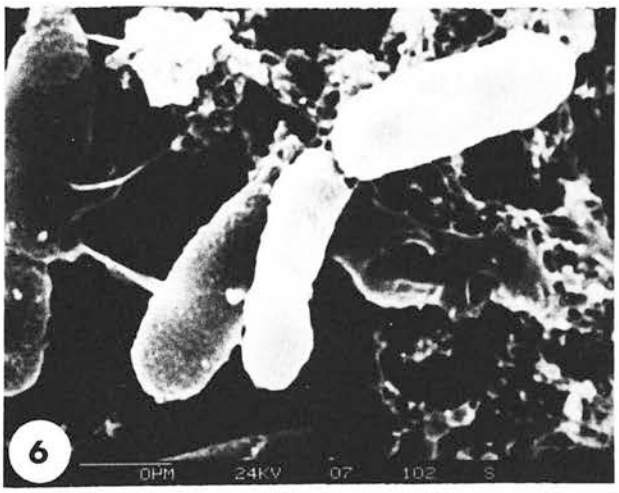
2PM 10KV 07 885 S


4
1PM 10KV 07 899 S


5
2PM 24KV 07 098 S


6
0PM 24KV 07 102 S

At 16h, microcolonies are more evident. Associated with them is a weblike sheet of polymer, embedding all the cells. Filamentous material can be seen at the periphery of the microcolony, as well as between individual cells. These polymeric strands are still comparatively thick and are more numerous than at 8h.

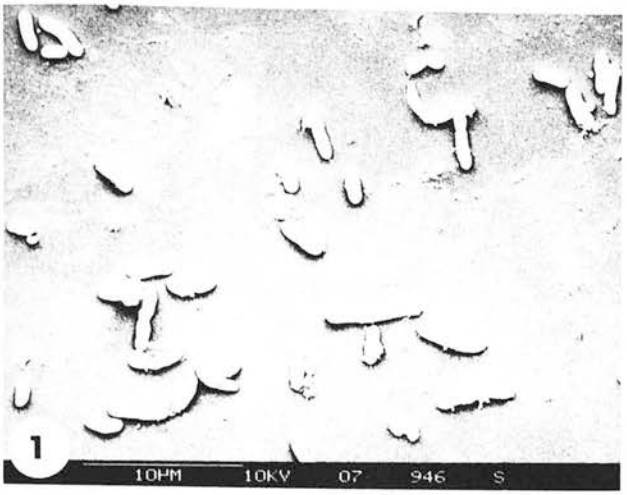
The amount of polymer has increased in relation to the increased number of cells observed adhering after 24h. Fibrillar sheets are shown to be extending both between cells, and cell and surface. Growth and division of the microcolony is both away from the surface as well as along the plane. The polymeric material does not appear to be adhering to the surface from any one site. Overall, the results show that for Strain S61 growing and attaching in media with excess carbon, there is a progressive build up of thick polymeric strands with time, leading to the formation of microcolonies enmeshed in a matrix of filaments and web-like mucoid material.

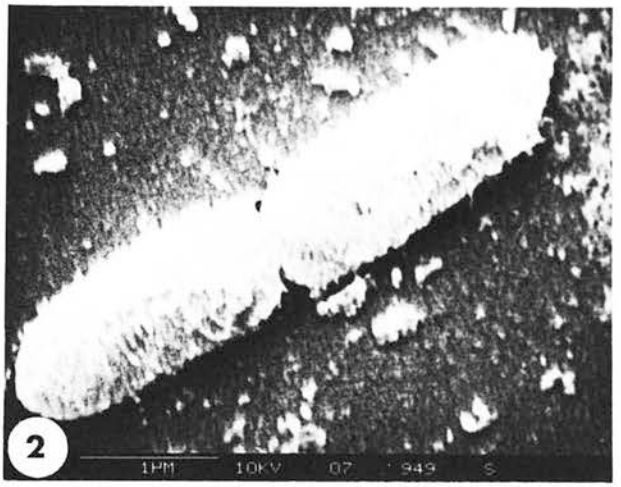
6.3 Attachment of S61: Carbon Limitation.

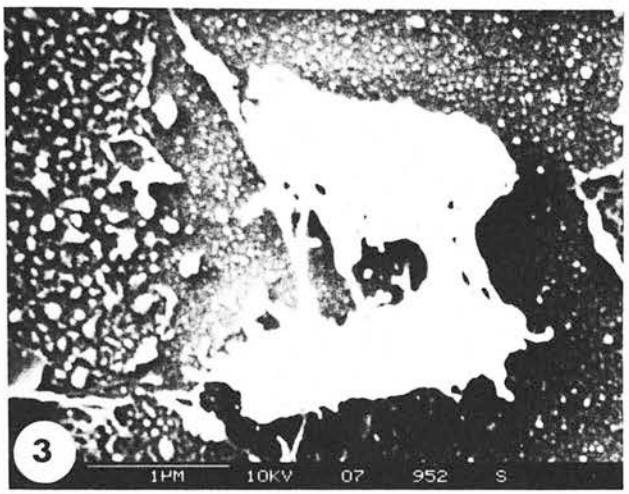
A careful consideration of the cell surface of Strain S61 grown under a carbon limitation (Plate 13), reveals that after 8h, thin microfibrils connect the cell to the glass surface. They appear to be randomly located about the cell, eliminating the possibility of being fimbriae or pili. As the high magnification shows (Plate 13.2) the microfibrils cannot be resolved to any finer detail; they remain as hair like projections protruding from the outer cell layers. Further examination of the cell surface reveals 'ridges' traversing the length of the cell. These may possibly be microfilaments of polymeric material, extruded from the cell. However, without better resolution no definite statement about their structure can be made.

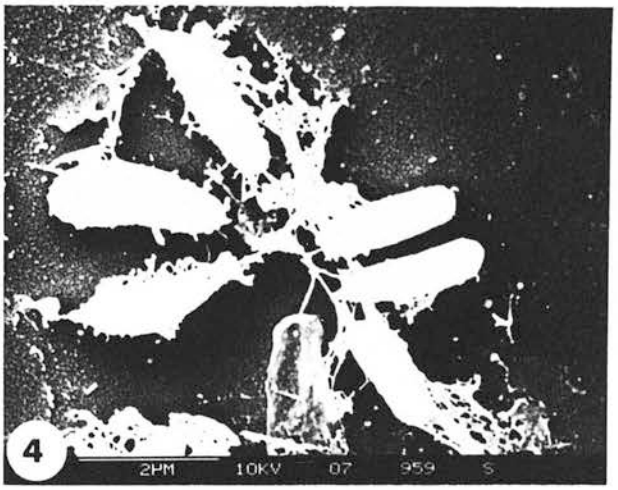
Cells adhering after 16h show an increased amount of associated polymer (Plate 13.3,4). This takes the form of polymeric strands which are fairly thick and numerous, extending both between cells and the surface. In addition, the bacteria appear to have a coating of polymer covering them. Unlike the cells in the previous experiment however (Plate 12.3), there is no evidence for the microcolonies being embedded in a sheet of polymer. The microcolony displayed in Plate 13.4 is arranged in a 'star' formation. Polymeric fibrils extend from each of the cells at various sites, with a central core

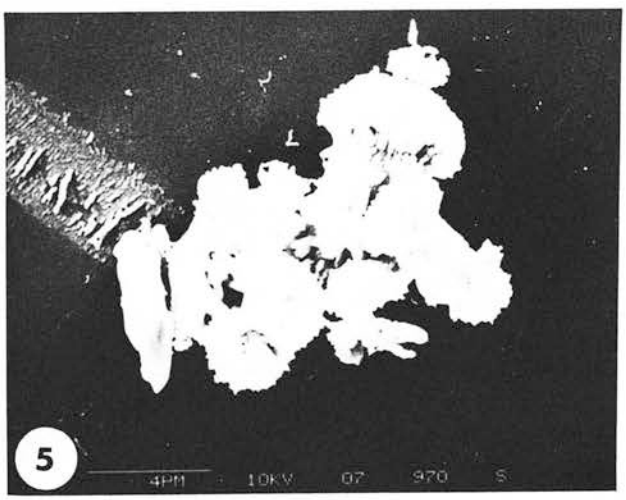
PLATE 13: SEM micrography of Strain S61 adhering to glass coverslips suspended in glucose limited YE media. Coverslips removed after 8h (1,2) 16h (3,4) and 24h (5,6).

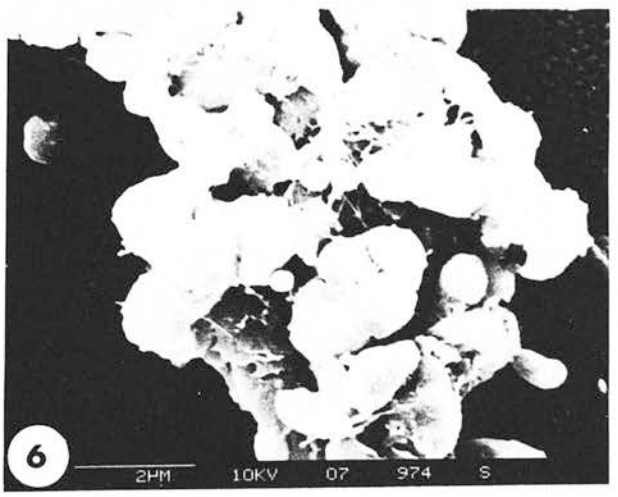

1
10PM 10KV 07 946 S


2
1PM 10KV 07 949 S


3
1PM 10KV 07 952 S


4
2PM 10KV 07 959 S


5
4PM 10KV 07 970 S


6
2PM 10KV 07 974 S

or 'plug' of polymer. This is not a typical microcolony formation and may possibly be due to an irregularity in the substratum surface.

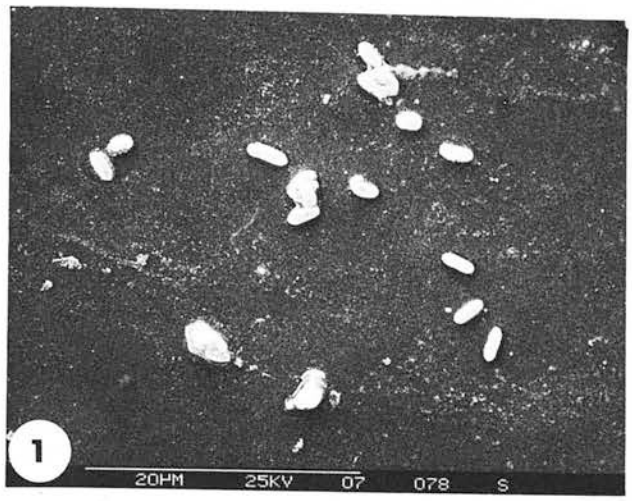
After 24h a change in the appearance of the microcolony has occurred. Whereas the cells described in Plate 12 have a fibrillar sheet embedding them, the cells illustrated in Plate 13.5,6 have no such material. Instead, the surface of the cells appears to be 'smooth', polymeric fibrils being evident only in the heart of the microcolony. The cell length has decreased by about 1μm from an original value of slightly greater than 3μm. This could indicate the process of 'Dwarfism' occurring. Novitsky and Morita (1976) demonstrated that different morphological forms of a marine vibrio spp depended upon the nutrient status of the environment. In low nutrient waters, copiotrophic bacteria tend to be starved and converted to dwarf forms. Despite the fall in viability, they retain a high degree of adhesiveness and show examples of polymer bridging (Dawson et al., 1981). Rounded, dwarf cells may therefore represent the cells found in the natural environment as laboratory conditions may make them increase in size due to rich media. At the 24h stage, the growth curve for Strain S61 under a glucose limitation (Section 2.2.3) has reached the point where cell viability is starting to decrease with a corresponding rapid decrease in residual glucose. It would thus seem feasible that the cells are beginning to starve and revert to a dwarf form.

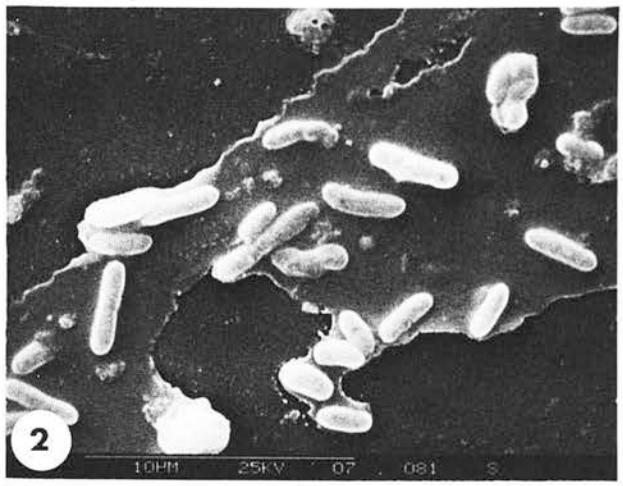
6.4 Examination of Unattached Bacteria.

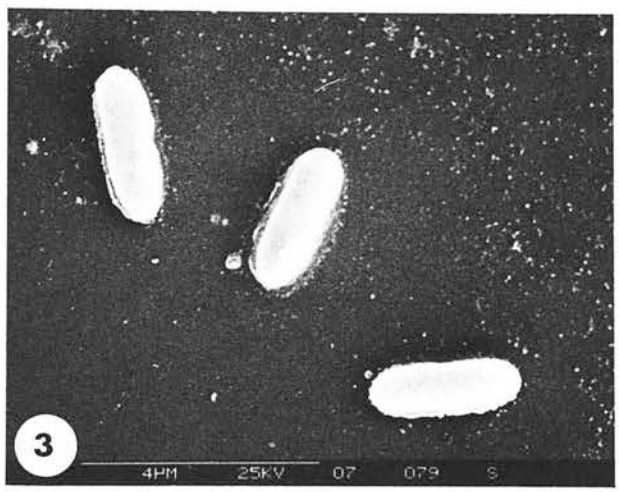
Electron micrographs comparing the cell surface of attached bacteria with those from the free living population are illustrated in Plate 14. A monolayer of planktonic bacteria was prepared by smearing a fixed sample of cells across a coverslip coated in polystyrene. The large positive charge generated on the glass surface is sufficient to hold the negatively charged cells. Results indicate that there is no evidence for the production of microfibrils by the unattached bacterial population (Plate 14.1,2,3). The cell surface is smooth compared to the fibrillar layers produced by cells grown in either a glucose limitation (Plate 14.4) or excess (Plate 14.5). This smooth appearance occurs irrespective of the carbon concentration.

The occurrence of polymeric fibrils involved in bacterial adhesion has been well documented (Gerchakov et al., 1977; DiSalvo &

PLATE 14: Cell surface comparison of Strain S61 planktonic population (1, 2 and 3) with attached cells under glucose limitation (4) and glucose excess (5).


1
20PM 25KV 07 078 S


2
10PM 25KV 07 081 S


3
4PM 25KV 07 079 S


4


5
2PM 10KV 07 894 S

Daniels, 1975). Cundell and Mitchell (1977) demonstrated that bacteria adhering on wooden surfaces after 16h were short rods (1μm long) anchored by polymeric filaments. After one week the bacteria were covered in copious amounts of polymer. The colonization and growth of bacteria on antifouling paints illustrated a variety of mucilage types involved in adhesion (Dempsey, 1981a and b). A continuous sheet of polymer was observed on non-toxic and Cu_2O based paint, whereas relatively small amounts of adhesive material was found on organotin based paints. These were in the form of fine polymeric strands. Similarly, Paerl (1975) described a variety of polymeric adhesives associated with the attachment of bacteria to suspended particles in both marine and freshwater. Fibrillar appendages, attached webbing and capsular secretions were all terms used to describe the polymeric secretions. Non-attached bacteria however, showed no evidence of this (Paerl, 1973).

The results presented here indicate that polymeric material is important for the build-up of microcolonies. This is in agreement with the ideas proposed in Section 3 from light microscopical studies. As cells grow and divide on a surface, more polymer is produced enabling microcolony formation to occur. Cells grown with an excess of carbon substrate form a web of polymer which embeds associated cells and extends outwards. The polymeric strands are therefore free to interact with other cells. When grown in a carbon limitation however, the cells round up when available glucose is used up. No polymer extends from the microcolony, polymeric fibrils being observed only between constituent cells. Because of the lack of polymeric fibres, microcolony formation will be restricted. This would partly explain the results observed by light microscopy. Polymeric fibres are important for microcolony formation, anchoring cells to each other and the surface.

Attachment of the cell appears to be independent of polymeric fibre production. The polymer is concerned not with the initial adhesion process but with the development of the microbial film. However, under a carbon limitation, microfibrils were observed to be intimately involved in the association between cell and substratum. When grown with an excess of carbon, their involvement may possibly be masked after 8h by the production of polymeric fibres. Both

energies of attraction and repulsion are functions of the radii of curvature of cell surface components. The magnitude of both decrease with decreasing radii, but those of repulsion decrease more. Hence, surface components with a very small radii will experience less repulsion and more attraction when approaching the substratum. The potential energy maximum (Fig 6) will be overcome, allowing the gap between the substratum and bacterium to be bridged.

The appearance of microfibrils on the surface of attached cells only, would suggest that they occur as a response to the substratum. Fletcher (1980b) reported that attachment was dependent upon both energy production and protein synthesis. The frequent decrease in effect of inhibitors added before introduction of cell to substratum indicated that time-dependent metabolic activity is required for firm attachment; that is, after the bacterium has initially encountered the substratum. This requirement however would appear to vary not only with the organism, but also with the nature of the substratum. The possibility of spontaneous attachment or even rapid adsorption onto a substratum would therefore remain a serious consideration.

In the natural environment, these polymeric structures may be involved in more than simply mediating adhesion. An increased surface area provides a region for ion exchange and the adsorption of nutrient molecules, acting in a manner similar to an ion exchange resin. It has been suggested that the large surface area binds extracellular enzymes thereby aiding the digestion of adsorbed nutrients. Other important functions of the mucilage for fouling bacteria include protection against predators and agressins.

As the literature stands, the nature of these microfibrils remains unclear. The possibility exists that they are modified fimbriae or pili, or that they are simply polysaccharide/glycoprotein structures. A question also remains as to whether or not they are a function of surface associated growth.

SECTION 7 Isolation of a Specific Cell Surface Receptor

7.1 Introduction

The involvement of specific cell surface receptors in bacterial adhesion has been well documented for cases of invasion and infection (Eshdat *et al.*, 1978; Firon *et al.*, 1983; Parry *et al.*, 1984). However, few examples have been reported for attachment in aqueous environments. Brown *et al.*, (1977) suggested the possible involvement of specific cell surface receptors for cells grown under conditions of glucose limitation. Bacteria were shown to attach in high numbers, with little EPS being produced. Moreover, in conditions of carbon excess, the reverse occurred; greater yields of polysaccharide resulted in reduced levels of bacterial attachment. The proposal was that specific surface receptors were available for adhesion when grown in glucose-limiting conditions but were saturated by the greater yield of EPS under conditions of glucose excess. Little is known about bacterial lectins and their possible role in adhesion, although examples of such compounds have been reported. The surface of *Streptococcus salivarius* is complex, carrying eight categories of functional groups of adhesions (Handley *et al.*, 1984). One of these, a fibrillar cell wall-associated glycoprotein, has been shown to be involved in the colonization of the human oral cavity (Weerkamp *et al.*, 1984). Some strains of motile *Aeromonas* produce lectin-like adhesins the activity of which can be inhibited by D-mannose (Atkinson *et al.*, 1983). The involvement of lectin-like receptors in adhesion may prove to be more common than was originally thought.

Previous results (Sections 3, 4 and 6) have indicated that the initial step in the adhesion process of Strain S61 is not mediated by EPS. Instead, a receptor in the outer membrane is thought to be involved. Inhibition of polysaccharide synthesis did not prevent adhesion, whereas treatments known to affect membrane proteins had a marked effect. The aim of this work described here was to determine whether a specific cell surface receptor is involved in adhesion, and to purify the resulting molecule.

7.2 Plate Count Experiments

7.2.1 Attachment to Polysaccharide Coated Surface

A pre-requisite for adhesion of any cellular material is the

prior accumulation of an adsorbed organic layer (Baier et al., 1968). In natural waters a variety of macromolecules exist which will adsorb onto the substratum. The source of these macromolecules is either as secondary products from living cells, or autolytic products from dead cells. Corpe (1970b) showed that bacterial adhesion to glass slides coated in polymer was significantly greater than to untreated slides. A similar experiment was carried out with Strain S61. Chemically clean glass slides were immersed in a 0.01% (w/v) solution of S61 EPS and removed after 1h. After air drying, the slides were suspended in YE salts and the attachment ability of S61 measured as previously described (Section 4). The results (Fig 38) show that there is a greater rate of attachment to the polymer coated slide, and a significant increase in the number of attached cells. Two possible explanations exist: (i) the adsorbed carbohydrate layer modifies the substratum by reducing the surface charge and the free energy, thereby making the surface more favourable for adhesion or (ii) the cells are able to interact specifically with the polymer on the surface. A close examination of the results from Section 3 indicates that before cells are observed to attach, the substratum adsorbs carbohydrate. This may include EPS secreted into the environment by the growing cells.

7.2.2 Sugar Inhibition Studies

Examples have been reported in the literature for mannose sensitive adhesins (Atkinson et al., 1983; Eshdat et al., 1978). If the cells in the above experiment are interacting specifically with adsorbed polysaccharide, addition of the monomeric sugar components in high concentrations may inhibit adhesion. Acid hydrolysis of the EPS isolated from Strain S61 revealed the presence of D-glucose, D-galactose, D-mannose, L-rhamnose and a uronic acid (Section 2.2.1). The attachment ability was measured using various concentrations of the component sugars in YE salts, counting the number of attached cells as before (Section 4). The results (not shown) revealed that adhesion of Strain S61 was inhibited by concentrations exceeding 0.3M for any sugar tested. However, adhesion did occur at 0.2M concentration (Fig 39). Galactose appears to have very little effect on adhesion, the number of attached cells remaining almost constant over the 5h sampling period. Attachment

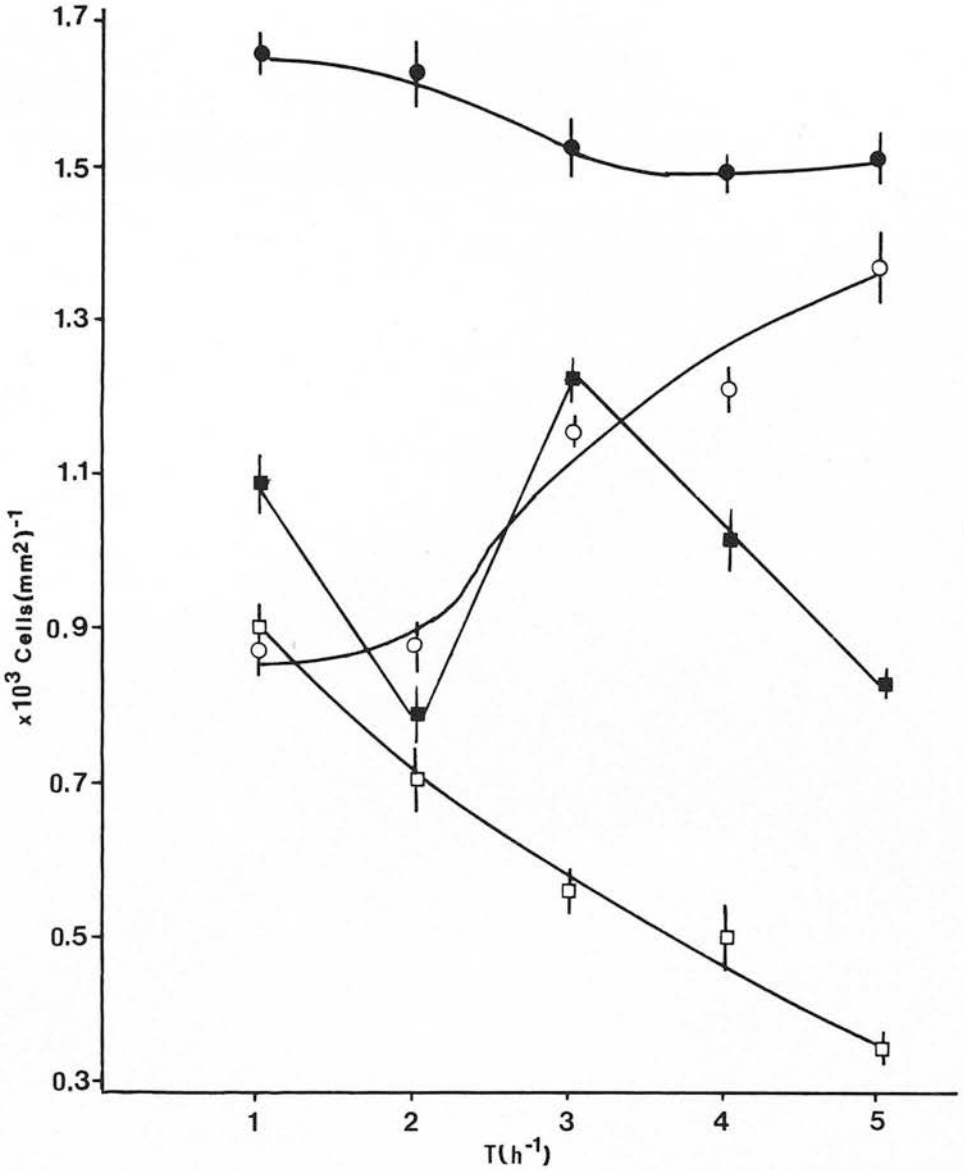


FIGURE 39: Effect of 0.2M sugar upon attachment of strain S61. (O) Glc; (●) Gal; (□) Man. and (■) Rha. Chemically clean glass slides suspended in YE salts containing the appropriate sugar, 30°C, 120rpm.

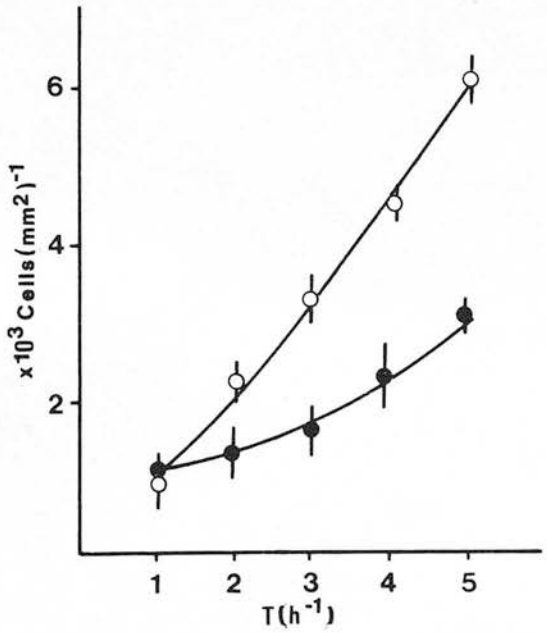


FIGURE 38: Attachment of strain S61 to (●) glass surface and (O) glass surface pre-coated in polysaccharide, suspended in YE salts. 30°C, 120rpm.

in the presence of glucose shows a similar response to the results expressed in the absence of glucose (Fig 24a). However, less than half the number of cells attached in the presence of glucose over a similar time interval. The effect of 0.2M mannose was to cause detachment of the cells. This indicates that a mannose receptor (or receptors) exists, which may in part be involved in adhesion. A variable response is shown by the presence of rhamnose.

7.3 Affinity Chromatography

Because of its high specificity, affinity chromatography has been widely used. The technique provides opportunities for the isolation of substances according to their biological function, and this differs radically from conventional chromatographic techniques which depend on gross physical and chemical differences between molecules. In the examples described below, the affinity columns consisted of immobilized sugars cross linked to a 6% agarose bead matrix.

7.3.1 Initial Separation

Purified outer membrane material, obtained by PBS-Sarkosyl isolation, was applied to an affinity column and eluted with phosphate buffer containing NaCl, until all unattached protein was removed. Subsequently, buffer containing 0.3M of the respective sugar was used to elute proteins specifically attached. The results, using immobilized glucose and galactose, are shown in Figure 40, the mannose column being described in Figure 41.

The elution profile using immobilized glucose reveals the presence of receptor molecules within the outer membrane material. However, no significant interaction is observed with the immobilized galactose column. Figure 41 indicates the presence of mannose specific receptors. The profile would suggest the possible presence of a number of receptors capable of interacting with mannose.

Initially, the outer membrane material was prepared by growing cells on a shaker (120 rpm) for 48h before harvesting. The elution profile however, was barely detectable for specifically absorbed material. Increased levels of absorption were found to occur if cells were grown for 24h at 120 rpm followed by a further 24h static growth. Eshdat et al., (1977) observed a similar response

FIGURE 40: Affinity chromatography of S61 outer membrane proteins on immobilized glucose (○) and galactose (●) columns. Elution buffer, containing 0.3M of the respective sugars (where indicated), was used to remove proteins specifically adsorbed.

FIGURE 41: Affinity chromatography of S61 outer membrane proteins on an immobilized mannose column. Elution buffer, containing 0.3M mannose (indicated), was used to remove proteins specifically adsorbed.

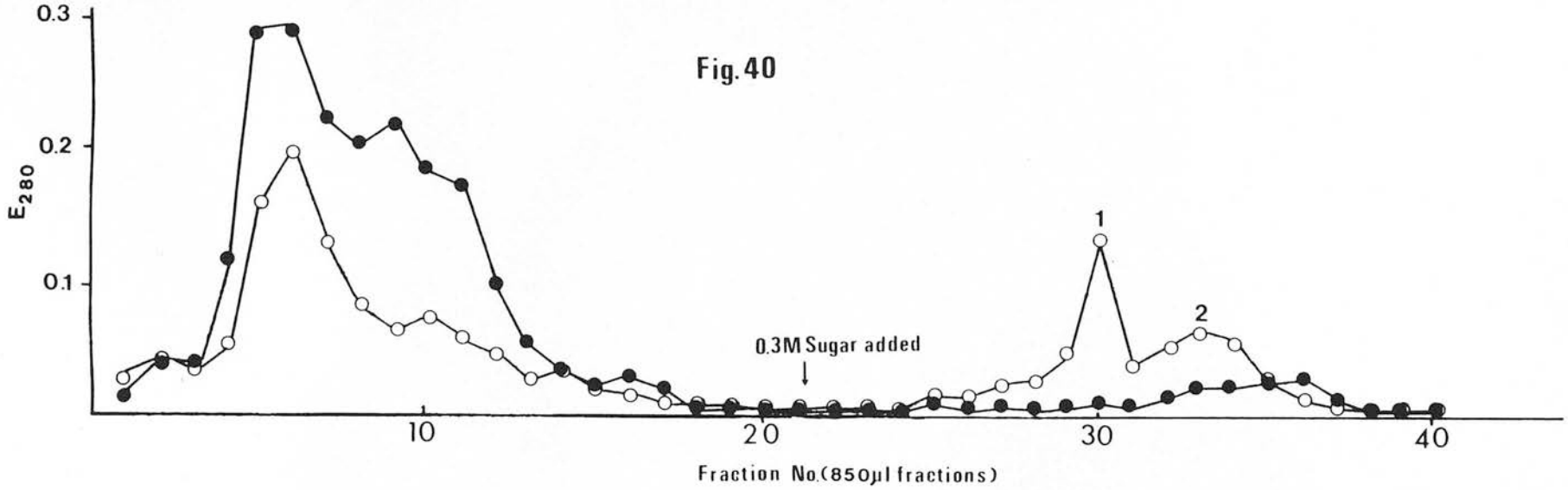

0.3
0.2
0.1
E_{280}
1
2
0.3M Sugar added
10
20
30
40
Fraction No.(850µl fractions)

Fig.40

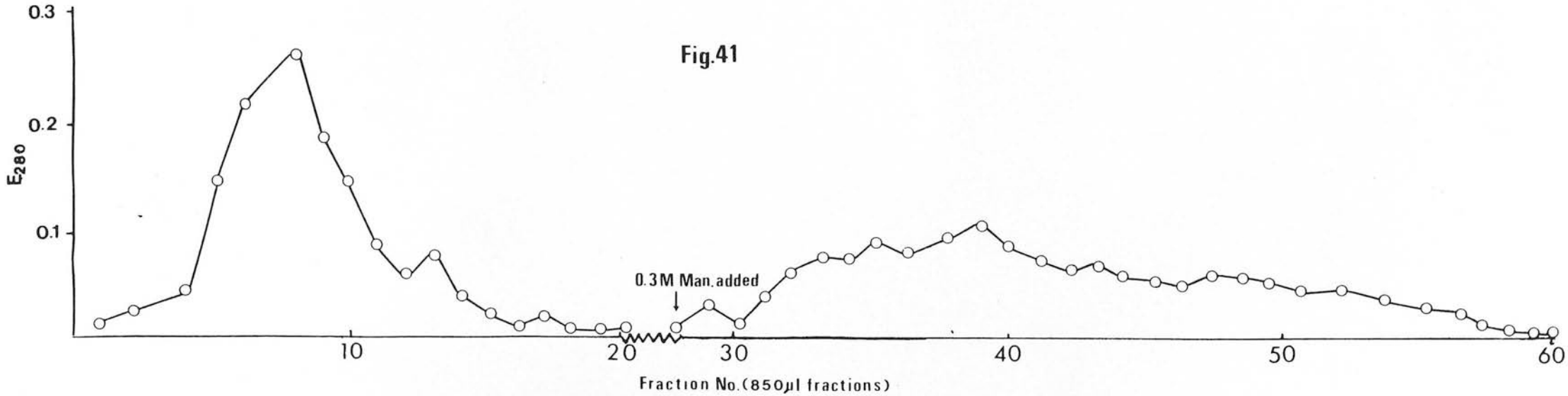

0.3
0.2
0.1
E_{280}
0.3M Man.added
10
20
30
40
50
60
Fraction No.(850µl fractions)

Fig.41

when isolating a mannose-sensitive lectin from E.coli.

7.3.2 Purification

The material eluted by the addition of 0.3M glucose to the buffer (Peak 1 and 2, Fig 40) was pooled, and dialysed against polyethylene glycol (5h) and distilled water (16h). This was re-applied to the immobilized glucose column, and eluted with a phosphate buffered glucose gradient (0.1M-0.5M). The resulting profile (Fig 42) reveals the presence of at least one group of glucose specific receptor molecules (Peak A) with the possibility that other receptors might exist (Peak B). The material from Peak A was concentrated, the degree of purity being observed by SDS-PAGE (Plate 15). As indicated, a protein band coresponding to a protein in the whole outer membrane prep. is observed. This has an approximate molecular weight of 89 000. Similar treatment to Peak B resulted in seven distinct bands appearing on the PAGE gel. The separation procedure was repeated, changing to the glucose gradient immediately the non obsorbed protein was eluted. Identical results were achieved. Thus, the results indicate the involvement of a cell surface receptor specific for glucose, in the initial adhesion step. Other glucose receptors may also exist, but as yet have not been identified. The 89K receptor has lectin-like properties but at present little is known about its biochemical and physical characteristics.

The mannose specific material from the initial column run was treated in a similar manner as above. However, material eluted by a 0.1 to 0.5M mannose gradient did not achieve any significant further separation (Fig 43). The result would suggest though, that mannose receptors do exist, but a better purification procedure would need to be devised to achieve a noteworthy separation.

Monosaccharide specific cell surface receptors possibly involved in the adhesion process have been identified. These receptors are believed to be involved in the initial step in adhesion, EPS being involved in the development of the microbial film. How common an occurrence receptor mediated adhesion is in the microbial world, and their relationship (if any) to the observed microfibrils is open to further investigation.

FIGURE 42: Purification of outer membrane glucose binding proteins (Peaks 1 & 2, Fig 40) on an immobilized glucose affinity chromatography column. Proteins eluted using a phosphate buffered glucose gradient (0.1M-0.5M), where indicated.

FIGURE 43: Separation of outer membrane mannose binding proteins on an immobilized mannose affinity column. Proteins eluted using a phosphate buffered mannose column (0.1M-0.5M), where indicated.

Fig. 42

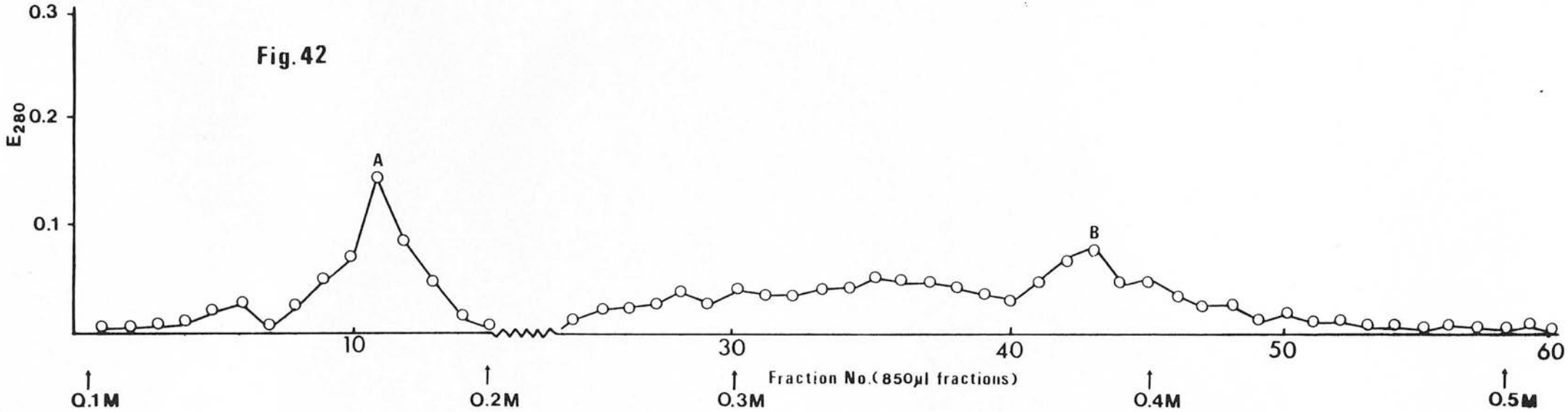
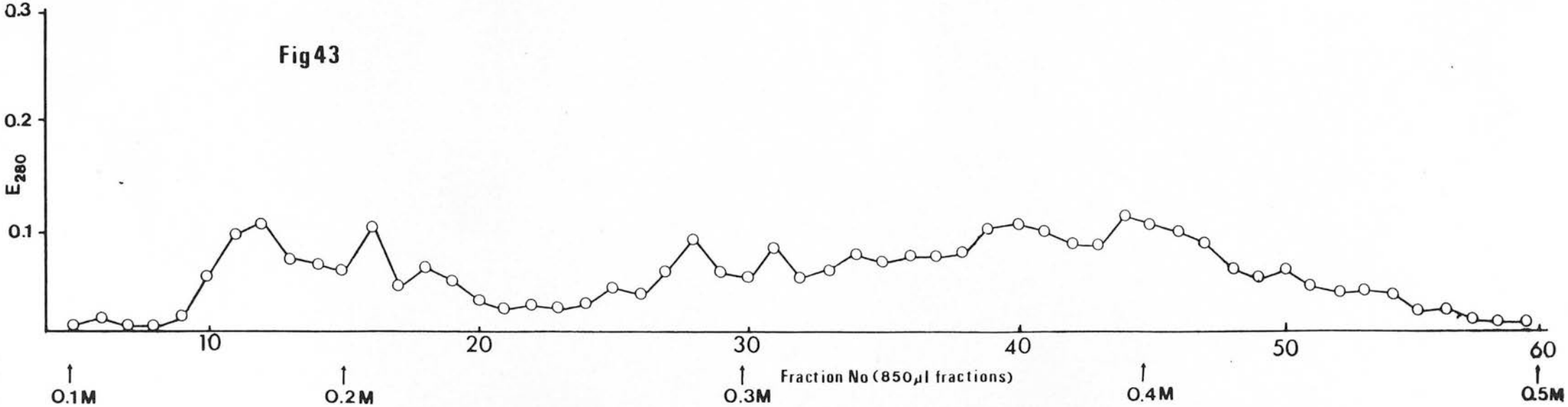

0.3
0.2
0.1
E_{280}
A
B
10
30
40
50
60
0.1M
0.2M
0.3M
0.4M
0.5M
Fraction No.(850µl fractions)

Fig 43


0.3
0.2
0.1
E_{280}
10
20
30
40
50
60
0.1M
0.2M
0.3M
0.4M
0.5M
Fraction No (850µl fractions)

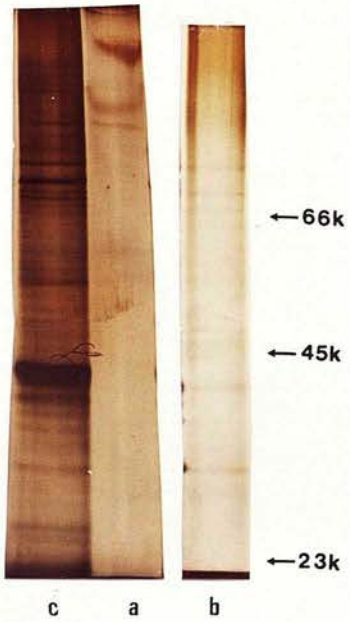


PLATE 15: PAGE-gel of glucose specific proteins isolated by affinity chromatography from S61 outer membrane (Fig 42). (a) Glucose specific protein corresponding to Peak A. (b) Glucose specific protein corresponding to Peak B. (c) Whole membrane prep. Molecular weight markers indicated.

CHAPTER FOUR: DISCUSSION

DISCUSSION

1.1 Introduction

Bacterial growth in many environments is associated with attachment to submerged surfaces. These include natural surfaces, particulate detritus, man made structures such as ship hulls and other micro or macro-organisms. The formation of attached microbial biofilms can lead to severe economic and ecological implications. Primary film formation by bacteria affects the subsequent attachment and development of larger organisms, resulting in the fouling and corrosion of submerged surfaces. However, bacterial adhesion is not always a detrimental process, as use can be made of immobilized cells in, for example, the treatment of wastewater. Attached bacterial populations are particularly significant in low nutrient waters, where the numbers of suspended organisms are low. The exposed solid/liquid interface provides an environment at which nutritional factors deficient in the surrounding fluid may be found. Nutrients may be more concentrated at the attachment surface through various adsorption mechanisms, making them more readily available to the attached population. Alternatively, the surface itself may be metabolized by the attached organisms. In either case, a submerged surface offers an ecological advantage to attached micro-organisms in an otherwise nutritionally depleted environment.

Despite increased attention in recent years, the exact mechanism of bacterial adhesion remains unclear. A number of modes of attachment have been described, including the involvement of specialized structures such as pili, fimbriae and holdfasts in *Caulobacter* and other stalked bacteria. However, most aquatic bacteria have no obvious means of attachment, and appear to 'stick' to the substratum by production of non-specific extracellular polysaccharides. The implications of EPS in the attachment process have been made by various authors (Jones *et al.*, 1969; Characklis, 1973; Fletcher & Floodgate, 1973; Geasey *et al.*, 1977), yet little is known about the actual role they play.

1.2 Isolation and Characterization of Adherent Freshwater Bacteria

Adherent, mucoid bacteria were isolated from glass surfaces suspended in a fast flowing river for 24h. In slow-moving fluids, shear forces generated are too small to stop the formation of adhesive bonds, whereas in faster moving fluids, the higher shear

is too great to allow most cells to adhere. Thus, organisms found attached in turbulent conditions will theoretically show an increased capacity for adhesion. The majority of bacteria isolated from the glass surface were found to be gram negative. However, population studies on the river revealed that this is more than likely to be due to the area in which the glass slides were suspended.

Analysis of the EPS isolated from adherent strains revealed no unusual structural component that could be associated with adhesiveness. Similar results were obtained for studies on marine bacteria (Sutherland, 1980), leading to the general assumption that the tertiary structure of the polymer is possibly involved.

1.3 Visualization of Adhesion

1.3.1 Light Microscopy

Detection of slime layers associated with both cell and substratum have relied heavily upon the use of electron microscopial techniques. Although structures involved in attachment can be identified, the techniques employed are subject to certain limitations. Use of the light microscope has previously been confined to enumeration and identification studies. However, by application of a novel staining technique, the involvement of EPS in the attachment process can be followed either *in vitro* or *in situ*. Quarternary ammonium salts are used to precipitate the polysaccharide which is subsequently stained by congo red intensified by Tween 80, the cells being stained by carbol fuchsin. The advantages of this technique are that it is quick and easy to perform; adhesion under a variety of situations can be followed, the results being expressed in a qualitative manner. More importantly, the results from laboratory studies appear to reflect events occurring in the natural environment.

Using both a capsulate organism (Strain S61) and a slime-producing organism (R1a), the involvement of EPS in the adhesion process was clearly demonstrated (Section 3). In both examples, carbohydrate material associated with the glass surface accumulated gradually over the initial 5-6h exposure, then increased rapidly over the remaining 10h. A few isolated bacterial cells were observed after 4h with little associated corbohydrate material. However, after 8h, microcolony formation started to occur, the aggregates being surrounded by a polysaccharide matrix.

The effect upon adhesion of treatments known to either prevent formation of the secondary and tertiary structure of the polysaccharide or inhibit production was studied using Strain S61. Previous results (Marshall *et al.*, 1971a; Fletcher & Floodgate, 1976) have shown the importance played by Ca^{2+} and Mg^{2+} ions in the ability of mucoid organisms to attach. When transferred to media deficient in both cations, cells were observed to detach from the test substratum, with a simultaneous disruption of the surrounding polymer. When grown in the absence of the two divalent cations, S61 was shown to produce more polymer in the surrounding medium than with Ca^{2+} and Mg^{2+} present. The amount of polymer adsorbed to the substratum however, was minimal. Cell attachment still occurred, but without the formation of microcolonies. These results suggest that cations (and particularly Mg^{2+} with strain S61) play an important role in both regulation of polysaccharide biosynthesis and maintenance of the tertiary structure. Further evidence for the involvement of cations was provided by the inhibiting effect of the chelating agent, EDTA. Recent proposals for the role of these cations in adhesion have included bridge formation between the negatively charged substrata and micro-organisms, stabilizing the structure of the EPS and causing precipitation of EPS in the space between a cell and substratum (Characklis & Cooksey, 1984).

A major difference was observed in the adhesion pattern using a non-mucoid isolate (Strain R3c) and a non-mucoid mutant of Strain S61 (designated NS61-20b). Both cell types were shown to adhere in relatively high numbers but without the formation of microcolonies. Little in the way of polysaccharide material was detected on the glass substratum, the attached bacteria being present individually or occasionally in groups of two or three cells. This would suggest that polysaccharide is not essential for adhesion, and that some other cell surface structure is involved. Polysaccharides do however have an important role in the adhesion process, that of microcolony formation. Growth and attachment of Strain S61 under glucose limitation provided further evidence for this proposal. Cell counts revealed a slight increase in attached numbers, without any associated polymeric matrix. Microcolony formation was absent under carbon limitation.

1.3.2 Scanning E.M.

The three-dimensional picture provided by a large depth of focus can be used by SEM to view an adherent population of cells. Interaction of bacteria with each other and with the substratum may be studied in finer detail than would otherwise be possible. However, greatest value from SEM studies can be achieved when used as a 'back-up' to other techniques. Examination of material by SEM alone is open to criticism as artifacts may be generated during sample preparation.

SEM micrographs of cells adhering under both carbon excess and limitation revealed the presence of microfibrils after 8h in the latter growth condition. These were not evident on cells grown in a glucose excess, or on the surface of either planktonic population. This would suggest a possible response to the surface by the cell. After 16 and 24h under the limitation, polymeric strands were observed to be associated with the cells, extending both between cells and surface. In contrast, cells grown in a carbon excess did not show microfibril formation. Instead, stout strands of polymeric material were observed. This however does not rule out the possibility that microfibrils were present, but were being masked by the polymeric material.

The involvement of microfibrils in the adhesion process has previously been suggested by Brown et al., (1977). When grown in conditions favouring polysaccharide production, the microfibril (or cell surface receptor) is saturated and not available for adhesion. However, when polysaccharide synthesis is inhibited or restricted, the maximum numbers of receptor sites are available for adhesion. In addition, microcolony formation appears to be dependent upon polymer production. Thus, for attachment to occur followed by growth and division on a surface, a fine balance must occur between the amount of EPS synthesized and the number of receptor sites available for adhesion. The chemical composition of the microfibrils observed by SEM is, as yet, unknown.

1.4 Specific Cell Surface Receptor

Results from the microscopical studies indicated the involvement of a surface receptor in the initial adhesive step. Further evidence for this idea was provided by studying the effect of an

EDTA wash upon the cells, and the effect of a polymer coated slide upon attachment. In the latter example, adhesion was enhanced. EDTA was however shown to inhibit cell adhesion, presumably by disrupting the outer membrane or removing some portion of it. Moreover, the results revealed by affinity chromatography (using purified outer membrane material) indicate the presence of at least one glucose specific receptor (M_r 89K daltons) and the possibility of less specific receptors for mannose and possibly other monosaccharides. The evidence presented here fails to distinguish between specific cell surface receptors and sugar binding proteins, with two exceptions. Sugar binding proteins in general, are located in the periplasm (eg. Stinson et al., 1977) and have a molecular weight ranging between 22 - 45K (Boos, 1974). The increased knowledge associated with mutation research could be made use of to a) selectively mutate the membrane protein and compare both adhesion and glucose uptake with the wild type, or b) obtain a non-adhesive mutant and compare the properties of the protein in each case. Recent reports in the literature indicate that adhesion mediated by cell surface glycoprotein molecules are relatively common (Atkinson et al., 1983; Irvin et al., 1984; Weerkamp et al., 1984). What relationship, if any, the glucose specific cell surface receptors have to the observed microfibrils is not known.

1.5 Effect of Culture Conditions

Although knowledge about attachment mechanisms is increasing, an understanding of factors influencing surface growth is also of considerable importance. In most environments there is a distribution between attached and free living bacterial populations, so that neither community is entirely predominant. Whether an individual bacterium becomes attached is dependent upon both its attachment mechanism and the environmental conditions. The physiology and attachment ability of Strain S61 was shown to be influenced by a variety of parameter changes. Cell concentration, temperature, glucose limitation and low salt concentration all affected the bacteria to some extent. Continuous culture is a useful tool by which to measure the changes in cell physiology under different environmental conditions and varying growth rates. Polymer production

and viscosity appeared to vary depending on the growth condition, whereas several of the outer membrane proteins generally increased in amount as the dilution rate increased. However, temperature had a more noticeable effect on both the amount and variability of the membrane profile. Alteration of growth conditions affects the physiology of the cell surface, which may subsequently influence the attachment ability of the organism in question. No generalities can be made though, as what applies to one organism under a given set of conditions, may not apply to another.

1.6 Conclusion

In conclusion, attachment and growth of Strain S61 to solid surfaces is believed to be carried out by the processes summarized in Figure 44. Attachment to the surface in question is mediated initially by specific cell surface receptors and, or including, microfibrils. A decreased radius of curvature provided by the microfibrils would suffice to reduce the energy of repulsion associated with the submerged substratum. Hence, surface components with a very small radius of curvature would experience less repulsion when approaching the substratum. Once attachment has occurred, growth and division on the surface occurs, associated with EPS synthesis. Only when polysaccharides are produced does microcolony formation occur. The role of the polymer would thus appear to be to anchor the cells both to the surface, and to each other, resulting in the formation of a biofilm. The term 'Glycocalyx' has often been applied to this cell/polymer matrix (Costerton et al., 1978; Bryant et al., 1984), a casual but misleading term. Other macromolecules besides polysaccharides and sugars are found within the matrix, including proteins, glycoproteins and a report of nucleic acids (Nishikawa & Kuriyana, 1968). The tertiary structure of the polymer is important for maintenance of the biofilm, though does not restrict the cells ability to adhere. Ca^{2+} and Mg^{2+} ions in turn are important for maintaining and regulating the structure and synthesis of the EPS.

Although slight progress has been made, many areas relating to microbial adhesion remain relatively obscure. The chemical composition of adhesive polymers has been studied, yet no complete study on the structure of these polymers has been reported (Suther-

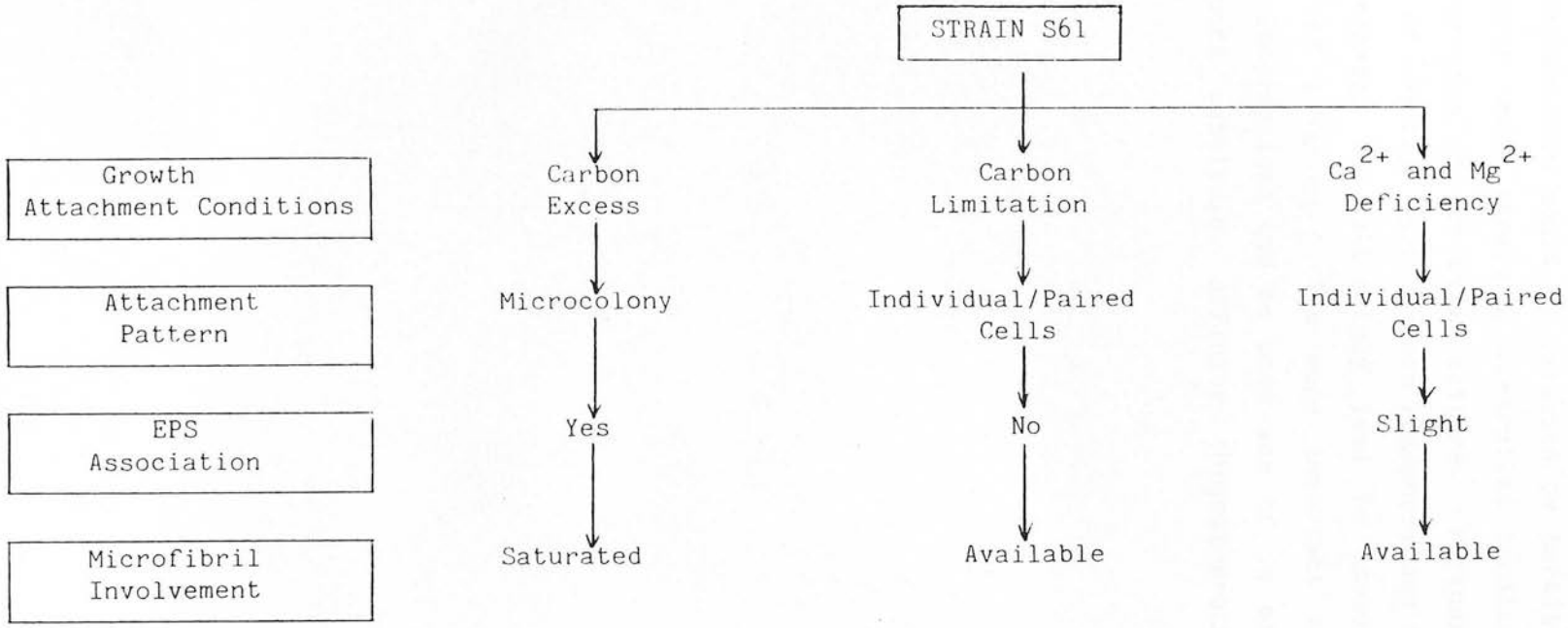


FIGURE 44: Summary diagram of attachment patterns and cell surface involvement in strain S61 under different growth conditions.

land, 1983a). It may prove possible to correlate physical properties with chemical structure. However, if organisms secrete more than one type of polymer, difficulties might arise in their resolution. The majority of studies to date have concentrated on the properties of purified polysaccharides and microbial strains. Perhaps more realistic information could be obtained by parallel studies using mixed cultures under carefully controlled conditions. Increased use could be made of continous culture. Further studies on the occurrence of specific cell surface receptors and their relationship to EPS synthesis may ultimately lead to novel techniques for prevention of adhesion. The more important aspects of cell-substratum interactions can be made use of in biotechnology (eg. solid support catalysis, affinity chromatography) with further effort.

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APPENDIX

APPENDIX 1

HPLC analysis of polysaccharide material was conducted as follows:

Polysaccharide hydrolysed, 1N H_2SO_4 at 100°C for 18h, neutralized with saturated barium hydroxide. The hydrolysate was passed through a Spherisorb SAX (5μ) column (Bibby Science Products) using 0.5N acetic acid as eluent. Triethylamine (1% v/v) was added to convert uronolactones to their acid form. Uronic acid free hydrolysates were subject to a two stage analysis.

1. Chromatography on a Zorbax NH_2 column with acetonitrile: distilled water (85:15) as eluent. This resulted in clear separation of 6-deoxyhexoses from hexose components.

2. Resolution of both the 6-deoxyhexoses and hexose material was achieved on a Biorad HPX-87P SCX column, eluting with distilled water at 70°C. Detection was by a Refractive Index Moniter.

APPENDIX 2

Published paper outlining the congo red staining technique for following bacterial adhesion by light microscopy.

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A staining technique for attached bacteria and its correlation to extracellular carbohydrate production

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Summary

Bacterial extracellular polysaccharides have been shown to be involved in the attachment process of cell to solid substratum. The method described here permits study of polysaccharides and their association with the substrate surface, using a novel staining technique for light microscopy. The method can be applied both in the laboratory and in situ and appears to be independent of polysaccharide composition and structure.

Key words: *Bacterial attachment – Exopolysaccharide – Light microscopy*

Introduction

Objects immersed in aqueous environments rapidly acquire an attached community of bacterial cells [1–2]. Various mechanisms of bacterial attachment have been described [3–4]. These include involvement of specialized structures such as fimbriae and pili or the holdfasts of *Caulobacter* sp and other stalked bacteria. A more common process in aquatic environments involves attachment associated with the production of a non-specific extracellular polymer which forms a slime covering the surface of the solid substratum. Isolated bacteria from the attached microbial flora of both freshwater and marine environments have been studied and shown to produce acidic polysaccharides involved in adhesion [5–8].

Detection of the slime layers has mainly been through electron microscopy, transmission electron microscopy (TEM) being used to identify the structures involved in attachment of cell to surface [5, 8–9]. One problem encountered is the generation of artifacts in the sample preparation and the lack of electron density

in the highly hydrated polysaccharide layer. Scanning electron microscopy (SEM), with its large depth of focus, provides a three-dimensional picture of the adherent population although it gives no indication of the chemical nature of the adhesive polymers.

The light microscope has found relatively little use because of its low resolution, thus limiting it to enumeration [1] and identification studies [10]. A novel staining technique has been developed permitting studies of bacterial attachment either in the laboratory or in situ using light microscopy. This simple staining procedure used in conjunction with a chemical assay reveals the association of polysaccharide material with adhering bacteria.

Material and Methods

Bacterial strains

The gram negative bacteria used in this study were isolated from a fast-flowing freshwater stream to the south-west of Edinburgh. All were maintained at 4°C on a semi-synthetic medium containing the salt solution described below with 1/10 concentration of yeast extract and casamino acids. All growth experiments were performed in a medium with the following composition ($g \cdot l^{-1}$): Na_2HPO_4 1.0, KH_2PO_4 0.3, K_2SO_4 0.1, NaCl 0.1, $MgSO_4$, $7H_2O$ 0.02, $CaCl_2$ 0.01, $FeSO_4$ 0.001, yeast extract (Oxoid) 1.0, casein hydrolysate (Difco) 1.0. Both liquid media and the corresponding agar were supplemented with 1% (w/v) glucose, autoclaved as a separate solution.

Adhesion studies in the laboratory

Chemically clean, sterile glass slides (75 × 25 mm) were suspended in 1 l culture in a 2 l Erlenmeyer flask, incubated at 30°C on a reciprocating shaker (120 rpm). Sampling was performed at regular intervals. On removal, slides were fixed and stained using a modification of a method developed by Nogrady and Michaud [11]. The surface of the slide was covered with 10 mM cetyl pyridinium chloride and air dried for 20–30 min. The slides were then fixed by gentle heating, allowed to cool, then stained for 15 min with a 2:1 mixture of saturated aqueous Congo red solution and 10% (v/v) Tween 80 solution. After careful rinsing, the slides were stained with 10% (v/v) Ziehl carbol fuchsin, followed by further rinsing and finally drying at 37°C. The quaternary ammonium salts are used to precipitate the polysaccharide which is then stained by the Congo red and intensified by the Tween 80. The cells are stained by the dilute carbol fuchsin solution. All chemicals were purchased from BDH Ltd., Poole, Dorset, England.

In situ studies

Chemically clean slides were suspended in the river and sampled at regular time intervals. Fixing and staining was carried out as for the laboratory material.

Analytical procedures

Sterile, chemically clean glass cover slips (18 × 18 mm) were suspended in the

river or in laboratory cultures. As a control, cover slips were also suspended in sterile, cell-free medium under the same conditions that were used for cultures in flasks. The cover slips were removed at regular time intervals, rinsed (3 ×) in distilled water, crushed and assayed for adsorbed carbohydrate using the Dubois phenol-sulphuric acid assay procedure [12]. D-glucose was used as standard. All measurements were made on a Zeiss (Oberkochen, F.R.G.) PMQ2 spectrophotometer.

Photography

All photographs were taken using a Leitz Orthoplan microscopy (100 ×, oil immersion) and camera attachment (Wetzlar, F.R.G.), with Kodak Ektachrome 160 film.

Results and Discussion

Bacterial growth in natural environments is often associated with attachment to surfaces, especially under limitation of specific nutrients [13]. The exposed surface serves as an area for concentration of the limiting nutrients, making them more readily available to the attached population than to the free-living cell types. The surfaces thus confer an ecological advantage to the attached microorganisms. Extracellular polysaccharides produced by aquatic bacteria are frequently associated with attachment to surface and to the subsequent growth of cells on these surfaces.

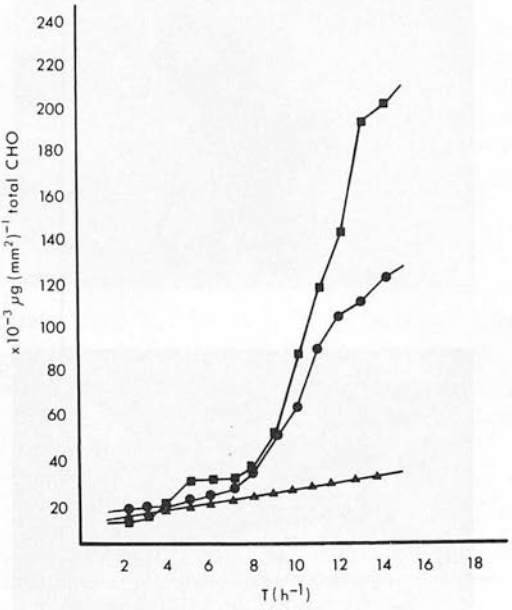

Fig. 1. Polysaccharide adsorption to glass surface. Mucoid strains S61 (■) and R1a (●) were allowed to adhere and grow on a glass cover slip suspended in the growth medium, in shake flasks at 30°C, 120 rpm, and the adherent polymer assayed as described. A control containing uninoculated medium was used for comparison (▲).

The systems used to test the involvement of polysaccharides in the attachment process were glass surfaces as described in the Methods, and Gram-negative bacterial isolates from a fast-flowing local river. The bacteria were chosen as being capsulate (strain S61) or slime-producing (R1a). Tests showed that there was a steady increase in the number of growing cells attached to the surface as indicated by the development of microcolonies, and an associated increase in the polysaccharide material associated with the surface. Typical results are illustrated in Fig. 1. Using either the capsulate or the slime-producing strain, there were similar results. Carbohydrate material associated with the glass surface accumulated gradually over the initial 5–6 h exposure, then increased rapidly over the remaining 10 h. In the control, there was a slight, non-specific adsorption of glucose to the glass surface.

The accumulation of cells and associated polysaccharide could be followed using the light microscope and the specific staining technique outlined in the Methods (Fig. 2). A few isolated bacterial cells were observed after 4 h exposure, with little associated carbohydrate material. However, after 8 h, microcolony formation started to occur, the aggregates being surrounded by a polysaccharide matrix. This increased in area after 11 h and also in depth, causing loss in resolution after 13 h. The accumulation of attached cells, through the production of non-specific polymers, has been described by Marshall et al. [14] as a three stage process. The first or reversible stage is the initial attraction of the cell to the surface, usually

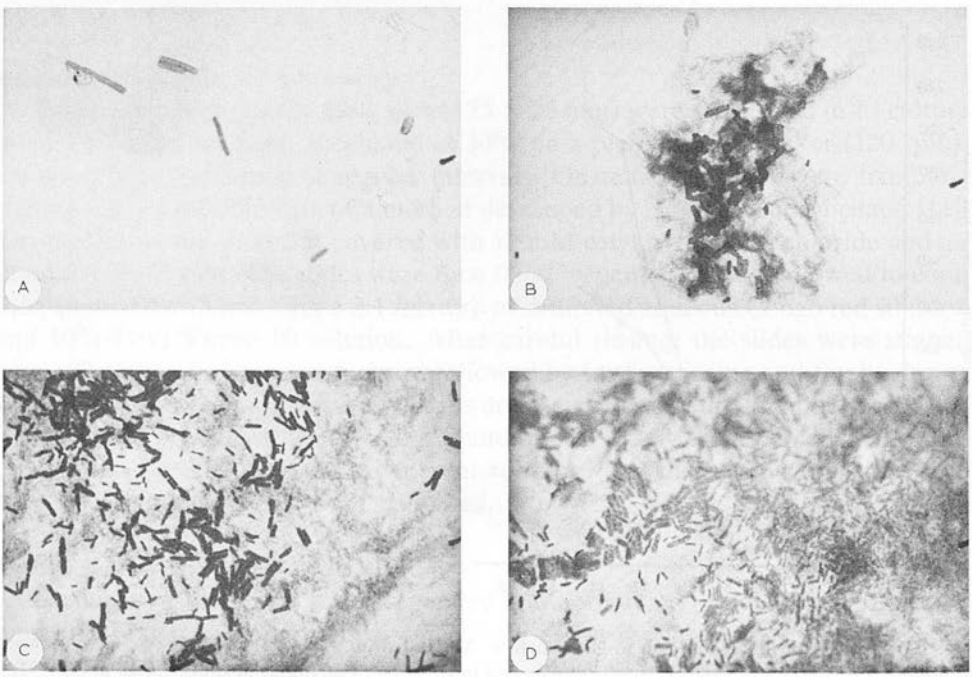

Fig. 2. Attachment and microcolony formation of a capsulate strain, S61. Chemically clean slides were immersed for (a) 4 h, (b) 8 h, (c) 11 h and (d) 13 h in shake flasks at 30°C, as described in the text.

through electrostatic forces. The second and third phases of growth and attachment are the irreversible (or time-dependent) stage and the phase of microcolony formation, respectively. Polymer is produced in these two later periods to enable the cells to attach firmly and irreversibly to the surface and allow them to grow and divide. These two stages are represented in the results shown in Figs. 1 and 2. There is little detectable difference in the carbohydrate levels found on cover slips immersed in cultures or sterile medium during the initial 4–5 h exposure (Fig. 1). This strengthens the concept of a requirement of a layer of organic material absorbed to the surface before colonization can occur [15].

In comparison with the accumulation of carbohydrate on the glass surfaces in the presence of bacteria synthesizing extracellular polysaccharides, use of the non-mucoid bacterial culture (R3C) yielded little carbohydrate on the glass surfaces (Fig. 3). The small amount formed is probably sufficient to attach the cells weakly to the glass, but may be insufficient to permit the bacteria to divide and form microcolonies. The bacteria were present on the surface individually in greatly reduced numbers, or occasionally in pairs. Production of exopolysaccharide thus seems to be an important factor in allowing the strains tested to adhere and subsequently to grow and divide on inert surfaces.

One of the problems in studies of microbial isolates from natural environments is the relation of observations made in the laboratory to actual in situ events [16]. To determine whether the results obtained in the laboratory studies reported here reflected the sequence of events in nature, clean slides and cover slips were sus-

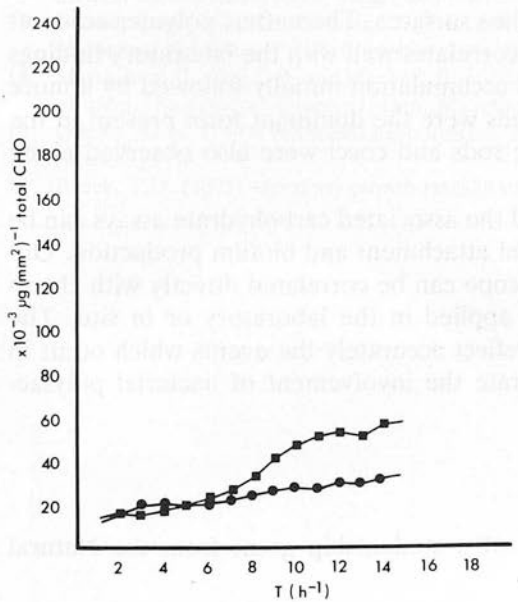

Fig. 3. Polysaccharide adsorption to a glass surface. This was performed exactly as in Fig. 1, using a non-mucoid strain, R3c (■). Again, a control containing uninoculated medium was used for comparison (●).

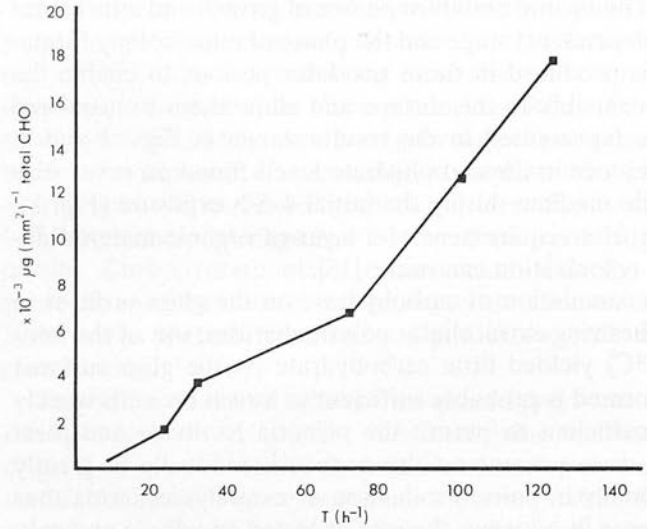

Fig. 4. Polysaccharide adsorption to a glass surface. Chemically clean glass cover slips were suspended in a fast flowing river at 9°C, for a period of 125 h, and assayed for adhered material as previously described (■).

pended in the river for periods of up to 125 h. As the mean river temperature was ~ 9°C, events could be expected to be much slower than in the laboratory. The results revealed (Fig. 4) that for 70 h there was a gradual increase in the amount of polysaccharide accumulated on the glass surfaces. Thereafter, polymer accumulation continued at a greater rate. This correlates well with the laboratory findings which also showed slow polysaccharide accumulation initially followed by a more rapid increase later in growth. Small rods were the dominant form present in the freshwater studies, though some larger rods and cocci were also observed along with associated polysaccharide.

The improved staining technique and the associated carbohydrate assays can be useful methods for the study of bacterial attachment and biofilm production. Observations made under the light microscope can be correlated directly with chemical assays and the techniques can be applied in the laboratory or in situ. The results from the laboratory appear to reflect accurately the events which occur in the natural environment and demonstrate the involvement of bacterial polysaccharides in the attachment process.

Acknowledgements

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